Nontoxic piperamides and their synthetic analogues as novel antifouling reagents

Xiang-Zhong Huang, Ying Xu, Yi-Fan Zhang, Yu Zhang, Yue Him Wong, Zhuang Han, Yan Yin, and Pei-Yuan Qian

Division of Life Science, The Hong Kong University of Science and Technology, Hong Kong SAR, China; Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, Yunnan University of Nationalities, Kunming, China

(Received 25 September 2013; accepted 27 January 2014)

Bioassay-guided isolation of an acetone extract from a terrestrial plant Piper betle produced four known piperamides with potent antifouling (AF) activities, as evidenced by inhibition of settlement of barnacle cypris larvae. The AF activities of the four piperamides and 15 synthesized analogues were compared and their structure–activity relationships were probed. Among the compounds, piperoleine B and 1-[1-oxo-7-(3',4'-methylenedioxyphenyl)-6E-heptenyl]-piperidine (MPHP) showed strong activity against settlement of cyprids of the barnacle Balanus amphitrite, having EC50 values of 1.1 ± 0.3 and 0.5 ± 0.2 μg l−1, respectively. No toxicity against zebra fish was observed following incubation with these two compounds. Besides being non-toxic, 91% of piperoleine B-treated cyprids and 84% of MPHP-treated cyprids at a concentration of 100 μM completed normal metamorphosis in recovery bioassays, indicating that the anti-settlement effect of these two compounds was reversible. Hydrolysis and photolysis experiments indicated that MPHP could be decomposed in the marine environment. It is concluded that piperamides are promising compounds for use in marine AF coatings.

Keywords: Piper betle; piperamides; antifouling compounds; activity relationship; hydrolysis and photolysis

Introduction

Biofouling is one of the most serious problems in the maritime and aquaculture industries. There are about 4,000 reported fouling organisms, more than 100 of which cause substantial damage to natural and artificial surfaces immersed in seawater (Dobretsov et al. 2006; Jorge et al. 2012). The accumulation of fouling organisms increases frictional resistance on ships’ hulls, increases the weight of buoys and oil platforms, blocks seawater pipes and reduces water exchange through aquaculture cages, and competes for space and food with cultured shellfish (eg Schultz et al. 2011; Fitridge et al. 2012; Sievers et al. 2013). To prevent and control marine biofouling, metallic, organometallic and organic compounds are commonly used as antifouling (AF) agents (Fusetani 2004; Konstantinou & Albanis 2004). Most of the AF compounds used in the past, as well as at the present time, are toxic to the environment. For example, although AF paints containing copper metal or copper compounds inhibit marine biofouling, they are harmful to non-target marine organisms (Wang et al. 2010). Concerns about the toxicity of AF biocides currently in use has stimulated research and development on non-toxic, non-heavy-metal-based natural AF agents (eg Qian et al. 2010, 2013; Li et al. 2012; Chandramouli et al. 2013).

In careful screening tests of terrestrial plants for natural AF biocides, a native plant from the southwestern region of China, the crude extract of Piper betle L. (Piperaceae), was shown to have potent AF activity (unpublished results). P. betle is widely distributed across South and South-East Asia and has been one of the most important plants throughout Asian history, ranking behind only coffee and tea in terms of daily consumption. It is estimated that nearly 600 million people consume this plant daily. It is particularly popular in India, China, Thailand, Indonesia and other South-East Asian countries (Guha 2006; Kumar et al. 2010). Its leaves and inflorescence are added to betel quid (BQ) for chewing. Chewing BQ is a widespread habit and has been credited with digestive, stimulant, carminative and other medicinal properties (Rowa & Hob 2009). It has recently been reported that leaves of P. betle exhibit many pharmacological effects including immunomodulator, antioxidant, antimicrobial, anti-allergic and anti-inflammatory functions (Singh et al. 2009; Al-Adhroey et al. 2010; Kumar et al. 2010). Previous chemical studies on this plant have reveal the presence of alkaloids, steroids, phenols and terpenes (Ghosh & Bhattacharya 2005; Rathee et al. 2006).

An extract of P. betle was evaluated for AF activity in this study due to its high therapeutic value and diverse
range of chemical compounds. Four structurally similar AF piperamides were subsequently isolated and identified from an acetone extract using bioassay-guided fractionation. The AF activity of these piperamides and 15 synthesized analogues was compared to determine structure–activity relationships among the compounds. The toxicity of two active compounds, piperoleine B and its analogue, 1-[1-oxo-7-(3′,4′-methyleneoxyphenyl)-6E-heptenyl]-piperidine (MPHP), as AF agents was evaluated using a zebra fish (Danio rerio) embryo toxicity assay and a recovery experiment with larvae of the barnacle Balanus amphitrite. The hydrolysis and photolysis of MPHP under abiotic conditions are also discussed. Overall, the results of this study are highly promising for the development of compounds for use in AF coatings.

Materials and methods

Plant material

Dry stems of P. betle L. were collected from the Baoshan District of Yunnan Province in China in October 2009. A voucher specimen (No. 20091020) was deposited at the School of Chemistry and Biotechnology, Yunnan University of Nationalities, China.

Chemicals

Silica gel (200–300 mesh) and silica gel GF254 were purchased from the Marine Chemical Industry Factory, Qingdao, China. RP-18 (40–70 μm) reverse-phase C18 silica gel was purchased from Merck, Darmstadt, Germany. Sephadex LH-20, CDCl3 was purchased from Sigma Chemical (Sigma Chemical Co., St Louis, MO, USA). Chromatographic grade acetonitrile was purchased from the Tianjin Chemical Reagent Factory (Tianjin, China). All other reagents were of analytical grade and were purchased from the Tianjin Chemical Reagent Factory (Tianjin, China) and Sigma Aldrich (Shanghai, China). Water was distilled twice.

Extraction and isolation of active compounds

The dried stems (6.5 kg) of P. betle were extracted with 70% acetone under reflux. The acetone extract was evaporated almost to dryness in vacuo, and the resulting mixture (760 g) was suspended in water before being successively partitioned with petroleum ether, ethyl acetate and n-butanol. The ethyl acetate phase was concentrated to produce a brown residue (170 g), which was separated into 11 fractions on a silica gel column using step gradient elution with CHCl3–MeOH (1:0–0:1). Fraction 2 (8.7 g) was applied to a silica gel column eluted with acetone–petroleum ether (1:16–1:8) to yield eight subfractions (Fr. 2.1–8). Subfraction 2.3 (3.6 g) was dissolved in acetone and recrystallized to obtain compound 2 (3.2 g). Subfraction 2.5 (1.1 g) was purified by a Sephadex LH-20 column using MeOH as a solvent before being subjected to an RP-18 silica gel column eluted with MeOH/H2O (60:40–80:20) to obtain compounds 1 (21 mg) and 3 (17 mg). Subfraction 2.7 (0.7 g) was chromatographed on an RP-18 silica gel column eluted with MeOH/H2O (65:35–80:20), and then purified by HPLC (MeOH/H2O, 69:31) to obtain compound 4 (23 mg).

Structure identification of compounds isolated from P. betle

The 1H and 13C NMR spectra were obtained on an Inova 500 FT-NMR spectrometer (1H, 500 MHz; 13C, 125 MHz, Varian Inc., Palo Alto, CA, USA) and a Bruker AV-400 spectrometer (1H, 400 MHz; 13C, 100 MHz, Bruker BioSpin, Zurich, Switzerland) in CDCl3 with tetramethylsilane (TMS) as the internal standard. Electrospray–ionization mass spectrometry (ESI-MS) data were recorded on a Finnigan LCQ-Advantage mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Structures of the compounds 1–4 were determined using NMR spectroscopy and mass spectrometry (MS). HPLC was carried out on an Agilent series 1200 HPLC instrument system with a diode-array detector (Agilent, Waldbronn, Germany).

Anti-larval attachment assay

Adult brood stocks of B. amphitrite were collected from Pak Sha Wan, Hong Kong (22°19' N, 114°16' E). Barnacle larvae were raised according to the method described by Harder et al. (2001). Cyprids were collected for the anti-larval attachment assay. Synthesized compounds from P. betle and 4,5-dichloro-2-octyl-4-isothiazolone-3-one (SeaNine 211) were dissolved in DMSO before being transferred into filtered seawater (0.22 μm filter pore size) to prepare test solutions at a range of concentrations from 0.06 to 20.0 μg ml−1. SeaNine 211 was used as a positive standard, as it is highly effective at controlling a wide range of fouling organisms (Li et al. 2012). One ml of test solution was added to each well of 24-well plates (Nunc, Rochester, NY, USA), after which about 15 cyprids were added to each well. The plates were incubated at 28°C in darkness and after 48 h, the numbers of settled and swimming larvae were counted under a dissection microscope. The number of settled larvae was described as a percentage of the total number per well. Four replicates were run for each sample. The EC50 (the concentration that inhibited larval settlement by 50% in comparison with the control) and LC50 (the concentration that caused 50% larval mortality in comparison with the control) were calculated as described in Zhou et al. (2009). The data were arcsine transformed and analysed by one-way analysis of variance (ANOVA). The zero values were assigned a value of n/4 (where n = number of larvae added to each
well) and analysed as described in Zar (1996). Data presented in the figures were not transformed.

Adults of *Hydoides elegans* and *Bugula neritina* were collected from Yung Shue O, Hong Kong (22°25′ N, 114°16′ E). The *H. elegans* larvae were reared for five days to a competent stage according to the method prescribed by Qian and Pechenik (1998). An anti-settlement assay with *H. elegans* larvae was performed with marine biofilm-coated plates following a method similar to one previously described (Carpizo-Ituarte & Hadfield 1998). Briefly, 24-well plates were suspended for five days in laboratory tanks supplied with running seawater in order to develop a natural biofilm. About 20 competent larvae were added to each biofilm well after the addition of 1.0 ml of test solution. The bryozoan larvae were collected according to the method described by Dobretsov et al. (2007). Newly released swimming larvae able to attach and metamorphose were used in the bioassays. About 20 larvae were added to wells containing a range of concentrations of the test solutions.

**Chemical synthesis of piperamide analogues**

The structure of piperamides can be divided into three main components, ie the methylenedioxyphenyl moiety, a conjugated side chain and an amide moiety. For the preparation of their synthetic analogues, modifications were made to the latter two components. Piperamide analogues were synthesized according to the method of Strunz and Finlay (1994) as detailed in Scheme S1 in Supplementary information (Supplementary information is available via a multi-media link on the online article webpage). Based on this method, reaction of cyclopentanone and cyclohexanone with piperonyl aldehyde in the presence of BF$_3$-etherate and ethylene glycol gave rise stereoselectively to the two key intermediates, the ethylene glycol ester of 6-(3′,4′-methylenedioxyphenyl)-E-5-hexenoic acid and 7-(3′,4′-methylenedioxyphenyl)-E-6-heptenoic acid. The two esters in acetone and aqueous LiOH were hydrolysed to give two carboxylic acids, respectively. The piperamides (5a–5e and 6a–6e) (Figure 2) were readily obtained from the carboxylic acids through condensation with appropriate amines in the presence of 1,1′-carbonyldimidazole. The unsaturated carboxylic acids were hydrogenated over 10% Pd-C and then amidated to give the corresponding saturated piperamides (7a–7e) (Figure 2). All the target compounds were subjected to ¹H and ¹³C NMR and ESI-MS analysis (NMR and MS data are shown in the Supplementary information) to determine the criteria for their chemical structures or purity (> 95%) before biological testing.

**Danio rerio (zebra fish) embryo toxicity assay**

Zebra fish embryos were bred as described by Westerfield (1995). Compounds were dissolved in fresh water to prepare a range of test concentrations before being transferred to 24-well plates. Five embryos at the cleavage stage (Kimmel et al. 1995) were then added to each well. The embryos were incubated with the compounds at 28°C and observed under a dissection microscope after 48 h.

**Recovery bioassay**

Piperoleine B and MPHP were dissolved in DMSO before being transferred into filtered seawater to prepare test solutions at a range of concentrations. One ml of test solution was added to each well of a 24-well plate (Nunc). About 5 μl of filtered seawater solution with about 15 cyprids were added to each well containing the test solutions. After 10 h most larvae remained alive. The treated larvae were then washed three times with fresh filtered seawater to remove the test solution. The larvae from each well were then transferred to a well of a new 24-well plate containing 1 ml of fresh filtered seawater. The plates were incubated for 48 h at 28°C in darkness. Controls were cyprids that had not been treated with test compound and had not therefore undergone the washing treatment. The number of settled and swimming larvae were counted under a dissection microscope after 48 h. The percentage settlement was calculated by expressing the mean of four replicates as a proportion of the total number of larvae in the well.

**Hydrolysis of MPHP**

Experiments were carried out according to methods described in ASTM (1993) and Zhou et al. (2007). Buffer solutions consisting of 20 mM sodium acetate were prepared and the pH was adjusted to pH 5, 7 and 9. Natural seawater (NSW), pH 8.1, was the same as that used in bioassays. Each of four different test solutions (450 ml) containing 1 mg l⁻¹ of MPHP (6b) was divided into nine test glass tubes (50 ml each), and the tubes were tightly capped. Immediately after the test solutions were prepared, the solutions in three tubes were loaded onto a Supelclean LC-18 column, pre-conditioned with methanol and double distilled water under reduced pressure. After loading with the test sample, the column was washed with 10 ml of double distilled water, followed by 2.5 ml of DMSO. HPLC analysis was performed using an Agilent series 1200 HPLC instrument (Agilent, Waldbronn, Germany) system equipped with a quaternary pump, a diode-array detector, an autosampler and a column compartment. Samples were separated on a Phenomenex Luna 5 Å C₁₈ 100A column (250 × 4.60 mm, 5 μm). The mobile phase consisted of acetonitrile (A) and water (B). A gradient program was used for elution: 0 min, 50% A; 30 min, from 50% A to 70% A; 10 min, from 70% A to 100% A. Flow rate, 1.0 ml min⁻¹; wavelength, 290 nm;
column temperature, 40°C. A volume of 20 μl of eluate was subjected to HPLC analysis to determine the initial MPHP concentration. The remaining six tubes were kept in an incubator at 50°C in the dark for a period of 3 or 7 days according to ASTM Procedure E895-89 (ASTM 1993). After each incubation period, three test samples processed as described above were subjected to HPLC analysis to determine the remaining concentration of MPHP. Each sample was analysed in triplicate using HPLC.

**Photodegradation of MPHP**

The test solution (450 ml) with 1 mg l\(^{-1}\) of MPHP (6b) was divided into nine test glass tubes (50 ml each). The tubes were tightly capped and then put into a XPA photochemical reacting apparatus (Nanjing Xujiang Electromechanical Factory, Nanjing, China), in which the tubes were irradiated using a 250 W high-pressure xenon arc lamp inside the device. The lamp has an emission in a wavelength range of 290–800 nm. The photochemical device was equipped with a water cooling unit to cool the xenon arc lamp and the reaction mixture, which was homogenized using a magnetic stirrer. A 50 ml sample of the test solution was removed from one of the tubes after 0, 0.25, 0.5, 1, 2, 3, 4, 8, 12 and 24 h, respectively. Samples were analysed by HPLC to determine the concentration of MPHP and its degradation products. There were five replicates for each fraction or compound. Each sample was injected into HPLC for analysis three times.

**Results and discussion**

**Structure and bioactivity of compounds isolated from P. betle**

The acetone extract from the stems of *P. betle* showed strong anti-settlement activity against cyprids of *B. amphitrite* with 71.9 ± 7.8% inhibition at 10 μg ml\(^{-1}\), compared with the control. Fractionation and further purification of the bioactive EtOAc fraction of the acetone extract yielded four AF piperamides (1–4) (Figure 1). The spectral data for these four compounds were in good agreement with those for compounds previously reported (Kiuchi, et al. 1988; Virinder et al. 1998). Thus, their structures were identified as four known piperamides, namely piperoleine B (1), piperine (2), piperdardine (3), and guineensine (4) respectively.

Compounds 1–4 belong to a class of piperamides with one methylenedioxyphenyl group, which are typically produced by three piper species, ie *P. betle*, *P. nigrum* and *P. longum* (Surrinder et al. 2000). Piperine (2), a well-known piperamide, has been reported to have sedative, hypnotic, anticonvulsant, antidepressant, antibacterial, antifungal, anti-tumour, and blood lipid reduction activities as well as being a cure for rheumatism and chronic bronchitis (Pei 1983). There are few reports relating to the pharmacological activity of the other three piperamides (1, 3, and 4). Compound 4 has been studied previously for its biocidal activity, showing activity against *Callosobruchus chinensis*, but not against *Toxocara canis* (Kiuchi et al. 1988).

In the present study the AF activities of compounds 1–4 are reported for the first time. The ability of these four compounds to inhibit larval settlement and their toxicity was assessed using cyprids of the barnacle *B. amphitrite*, larvae of the polychaete *H. elegans* and larvae of the bryozoan *B. neritina* (Table 1). Piperoleine B and piperine had low EC\(_{50}\) values against the three fouling organisms and high LC\(_{50}/EC_{50}\) ratios, which indicate that the two compounds not only have strong broad-spectrum anti-larval settlement activity, but also show low toxicity to the species tested. In particular, the anti-larval settlement activities of piperoleine B against the three fouling species were higher than those of SeaNine-211. An initial study of the structure–activity relationships of the piperamides synthesized or isolated from plants showed that the

![Figure 1. The structure of compounds 1–4 isolated from the stems of *P. betle*: piperoleine B (1), piperine (2), piperdardine (3) and guineensine (4).](image-url)
Bioactivity of this type of compounds was also related to the length of the alky chain and the nature of the amine moiety besides the methylenedioxyphenyl moiety (Kiuchi et al. 1997). To search for more efficient, environmentally friendly and lower-cost AF agents than piperoline B, 15 analogues related to piperoline B (Figure 2) were synthesized. Their activities against settlement of cyprids of *B. amphitrite* were investigated.

### Bioactivity of related compounds

The anti-settlement activity of piperamide analogues containing modified side chains was evaluated using a barnacle settlement bioassay (Table 2). Bioactivity data for piperamides isolated from *P. betle* or synthesized showed that among piperamides with various amine moieties, piperidine amides (compounds 1, 5b, 6b and 7b) exhibited the highest anti-larval efficacy with EC$_{50}$ values ranging from 0.5 to 1.5 μg ml$^{-1}$ (Tables 1 and 2). The data indicated that the piperidine group was the optimal terminal group as a substituent at the N-terminal. The results also showed that bioactivity of piperamides with either the piperidine group or the pyrrolidine group was stronger, whereas those with the piperazine group, morpholine group or the N-isobutyl group had significantly weaker bioactivity, which indicates that increases in bioactivity appear to be related to the lipophilicity of the amine moieties. Kiuchi et al. (1992) reported that amides of polar cyclic amines such as morpholine, piperazine and pyrrolidine showed stronger activity against *Toxocara canis* larvae than piperidine amides. This is in contrast to the present results and suggests that the anti-larval settlement activity of the compounds may work through a different mechanism.

A previous study (Kiuchi et al. 1997) determined the nematocidal activity of piperamides with various chain lengths against *T. canis* larvae and suggested that their nematocidal activity was dependent on alky chain length. In a series of homologues, bioactivity reached a maximum at an optimal chain length, the chain length becoming greater with a more polar amine moiety and shorter with a less polar amine moiety. The data were analysed in relation to the log $P$ values of the compounds and were attributed to the hydrophobic/hydrophilic balance. The alky chain lengths of the most bioactive homologues were C$_7$ alky chain for the piperidine amides, C$_{11}$ alky chain for the pyrrolidine amides and C$_{13}$ alky chain for the N-methylpiperazine amides, respectively. Among piperamide analogues with the piperidine group, MPHP (6b) containing a C$_7$ alky chain showed the strongest anti-larval settlement activity with EC$_{50}$ values of 0.5 ± 0.2 μg ml$^{-1}$ in the bioassay with cyprids of *B. amphitrite*. This is in good agreement with the case of the piperidine amides on *T. canis* larvae (Kiuchi et al. 1997). Furthermore, saturation of the double bond linked with methylenedioxyphenyl group had no obvious effect on the anti-larval settlement activity (5a and 7a, EC$_{50}$ = 2.3 ± 0.5 and 2.5 ± 0.6 μg ml$^{-1}$; 5b and 7b, EC$_{50}$ = 1.3 ± 0.3 and 1.5 ± 0.4 μg ml$^{-1}$). The activity was weakened by introduction of the double bond at the C-2 and C-4 positions and reduction of the double bonds at the C-6 positions (compound 3, EC$_{50}$ = 4.2 ± 1.3 μg ml$^{-1}$). Conjugation between the methylenedioxyphenyl moiety and the amine moiety by two double bonds was characteristic for piperine (2), which also exhibited obvious activity (EC$_{50}$ = 1.4 ± 0.6 μg ml$^{-1}$) in the bioassay.

### Toxicity of piperamides to zebra fish embryos

The two most active piperamides, piperoline B (1) and MPHP (6b), were assessed for their toxicity against zebra fish embryos. The LC$_{50}$ values of piperoline B and MPHP were 7.6 μg ml$^{-1}$ and 8.1 μg ml$^{-1}$, respectively. In both cases, live fish embryos did not show any obvious phenotypic changes in comparison with the control fish when observed at 48 h post-fertilization (hpf) and 60 hpf.

### Recovery of larvae treated with piperamides

The recovery experiments were designed to determine whether swimming larvae retained the ability to complete settlement and metamorphosis after removing the piperamides from the testing chamber. After exposure to

| Compound          | Fouling species | EC$_{50}$ (μg ml$^{-1}$) | LC$_{50}$/EC$_{50}$ |
|-------------------|-----------------|--------------------------|----------------------|
| Piperoline B      | *B. amphitrite* | 1.1 ± 0.3**              | > 86.9               |
|                   | *H. elegans*    | 1.0 ± 0.4**              | > 49.5               |
|                   | *B. neritina*   | 0.7 ± 0.2**              | > 14.7               |
| Piperine          | *B. amphitrite* | 1.4 ± 0.6**              | > 71.4               |
|                   | *H. elegans*    | 1.3 ± 0.8**              | > 38.5               |
|                   | *B. neritina*   | 1.6 ± 0.7**              | > 30.3               |
| Pipedardine       | *B. amphitrite* | 4.2 ± 1.3**              | > 24.0               |
|                   | *H. elegans*    | 7.3 ± 1.6**              | > 13.7               |
|                   | *B. neritina*   | 8.5 ± 2.1**              | > 11.7               |
| Guineensine       | *B. amphitrite* | 16.6 ± 2.9*              | > 12.1               |
|                   | *H. elegans*    | > 20.0                   | > 5.8                |
|                   | *B. neritina*   | 5.7 ± 1.5**              | > 17.6               |
| ScaNine-211       | *B. amphitrite* | 1.9 ± 0.4**              | –                    |
|                   | *H. elegans*    | 2.1 ± 0.6**              | –                    |
|                   | *B. neritina*   | 2.5 ± 0.7**              | –                    |

EC$_{50}$ = the concentration of a compound that inhibited larval attachment by 50% in comparison with the control. LC$_{50}$ = the concentration that caused 50% larval mortality in testing vessels in comparison with the control.

*p < 0.05; **p < 0.01 (one-way ANOVA). There were no significant differences among replicates at a significance level of 0.01.

### Table 1. Anti-larval settlement activity of the four piperamides, piperoline B (1), piperine (2), pipederidine (3), and guineensine (4) isolated from *P. betle* against three major fouling species.
Figure 2. The structure of the synthetic piperamide analogues (5a–5e, 6a–6e, and 7a–7e).

Table 2. Anti-larval settlement activity of piperamide analogues (5a–5e, 6a–6e, and 7a–7e) synthesized against B. amphitrite.

| Compound | EC$_{50}$ (µg ml$^{-1}$) | LC$_{50}$/EC$_{50}$ | Compound | EC$_{50}$ (µg ml$^{-1}$) | LC$_{50}$/EC$_{50}$ |
|----------|--------------------------|---------------------|----------|--------------------------|---------------------|
| 1a       | 2.3 ± 0.5$^{**}$         | > 21.7              | 2d       | 5.2 ± 1.8$^{**}$         | > 19.2              |
| 1b       | 1.3 ± 0.3$^{**}$         | > 38.5              | 2e       | 21.6 ± 3.6$^{**}$        | > 4.6               |
| 1c       | 11.0 ± 2.5$^{*}$         | > 9.0               | 3a       | 2.5 ± 0.6$^{**}$         | > 20.0              |
| 1d       | 7.9 ± 1.9$^{*}$          | > 12.7              | 3b       | 1.5 ± 0.4$^{**}$         | > 33.3              |
| 1e       | 23.1 ± 3.8$^{*}$         | > 4.3               | 3c       | 12.3 ± 3.1$^{**}$        | > 8.1               |
| 2a       | 1.9 ± 0.4$^{**}$         | > 26.3              | 3d       | 8.4 ± 1.3$^{**}$         | > 11.9              |
| 2b       | 0.5 ± 0.2$^{**}$         | > 40.0              | 3e       | 24.3 ± 4.3$^{**}$        | > 4.2               |
| 2c       | 10.3 ± 2.1$^{*}$         | > 9.8               | 3f       | 15.6 ± 3.2$^{**}$        | > 11.9              |

EC$_{50}$ = the concentration of a compound that inhibited larval attachment by 50% in comparison with the control. LC$_{50}$ represents the concentration that caused 50% larval mortality in testing vessels in comparison with the control.

$^*$p < 0.05; $^{**}$p < 0.01 (one-way ANOVA). There were no significant differences among replicates at a significance level of 0.01.
piperoleine B for 10 h, the recovery bioassay was used to calculate the percentage of metamorphosed larvae of *B. amphitrite* (Figure 3). Specifically, after exposure to 100 μM piperoleine B solution for 10 h, 91% of the treated larvae were able to settle and complete their metamorphosis. When treated for 10 h with 100 μM MPHP, 84% of larvae regained the ability to settle and metamorphose after being removed from the MPHP solution. The results suggest that the effect of piperamides on cyprids of *B. amphitrite* is reversible, and that piperamides do not harm larvae, which is similar to capsaicin, another AF agent (Torsten2007).

**Hydrolysis of MPHP**

Information on the environmental fate of a potential AF compound is important for regulatory purposes (Zhou et al.2007). In this study, the hydrolysis of MPHP was investigated according to the ASTM protocol (ASTM 1993). As shown in Table 3, the concentration of MPHP was significantly reduced after it had been stored at 50°C for one week. Approximately 31.5, 27.8, 13.8 and 23.3% of MPHP was decomposed in pH 5.0, 7.0 and 9.0 buffer solutions, and in 0.2 μm filtered NSW (pH 8.1), respectively. Hydrolysis at 50°C for 7 d is equivalent to hydrolysis at 25°C for six months according to the ASTM protocol (ASTM 1993). It was also observed that the change in MPHP concentration after incubation for 3 d was similar to that after incubation for 7 d. There was a slightly greater decomposition at lower pH levels than at higher pH levels, suggesting that degradation through hydrolysis would be slower under alkaline environments. Two new peaks, representing the main degradation products of MPHP (retention time of 7.5 and 8.8 min) in buffered solutions with different values of pH were detected during the hydrolysis study. From the ESI-MS spectrum of peak 2 (tR 8.8 min), this compound was identified as 7-(3′,4′-methylenedioxyphenyl)-6E-heptenoic acid from the ions at m/z 247.1 [M–H]– and m/z 265.1 [M + H2O – H]–. Peak 1 was unidentified.

**Photodegradation of MPHP**

Photolysis dynamics fitted with pseudo-first-order kinetics and a plot of the natural log concentration vs time should produce a straight line (ASTM 1993). Thus, first-order kinetics were applied to explain the photolysis rates of MPHP during irradiation by a high-pressure xenon arc lamp in the four test solutions. As shown in Table 4, the first-order rate constant (K) of MPHP calculated using the data during the period 0–12 h was 0.080, 0.060 and 0.072 h–1 for the buffered solutions at pH 5, 7 and 9, respectively. From the value of K, the half-life (T1/2) was 8.6, 11.5, and 9.7 h for the buffered solutions at pH 5, 7 and 9, respectively. It can be seen that both acidic pH and alkaline pH enhance the photolysis of MPHP. In addition, the concentration of MPHP in the NSW sample (pH 8.1) also decreased under photolysis (Table 4). The photolysis rate constant of MPHP in the NSW sample for the first 12 h was calculated to be 0.090 h–1. The half-life was estimated to be 7.7 h, which is lower than that of MPHP in the other three buffered solutions. The photodegradation of MPHP in five different conditions showed similar behaviour, with the production of peak 1 and 7-(3′,4′-methylenedioxyphenyl)-6E-heptenoic acid, which was similar to degradation by hydrolysis. Further studies are required to clarify the degradation pathway of MPHP in natural environments, as well as to identity peak 1.

Table 3. Hydrolysis of MPHP in the four test solutions at 50°C in the dark.

| Aqueous solution | Initial concentration (mg l–1) | Concentration after 3-days | Concentration after 7-days |
|------------------|-------------------------------|----------------------------|---------------------------|
| Buffer at pH 5   | 0.92 ± 0.01                   | 0.74 ± 0.03**              | 0.63 ± 0.06**             |
| Buffer at pH 7   | 0.90 ± 0.02                   | 0.78 ± 0.04*               | 0.65 ± 0.04**             |
| Buffer at pH 9   | 0.94 ± 0.01                   | 0.88 ± 0.02*               | 0.80 ± 0.03*              |
| NSW (pH 8.1)     | 0.90 ± 0.02                   | 0.83 ± 0.02*               | 0.69 ± 0.02**             |

MPHP concentrations are denoted as the means ± SD (for n = 3). The statistical difference is indicated at the significance level of: * p < 0.05; ** p < 0.01, calculated using one-way ANOVA.
Acknowledgements

piperamides could be considered as potential AF levels in marine environments. The results suggest that seawater, suggesting it would not accumulate to high indicated that signiHcance.

Table 4. Photolysis of MPHP in the four test solutions (0–12 h).

| Test solution          | k(h−1) | R²    | T1/2(h) |
|------------------------|--------|-------|---------|
| Buffer at pH 5         | 0.080  | 0.9803| 8.6     |
| Buffer at pH 7         | 0.060  | 0.9557| 11.5    |
| Buffer at pH 9         | 0.072  | 0.9454| 9.7     |
| NSW (pH 8.1)           | 0.090  | 0.9606| 7.7     |

The data for 24 h incubation were not included due to the long interval from the former sampling time (12 h). Correlation coefHcient was shown as R². Half life (T1/2) of MPHP calculated based on the linear correlation.

Conclusion

In this study, four known bioactive piperamides were isolated from an extract of P. betel. Among the four piperamides and the synthesized analogues, piperoleine B and MPHP exhibited strong anti-larval settlement activity and low levels of toxicity against marine fouling organisms and zebra iSh; their effect on cypris larvae of B. amphitrite was reversible at concentrations up to 100 μM. Hydrolysis and photolysis of MPHP indicated that signiHcant degradation occurred in natural seawater, suggesting it would not accumulate to high levels in marine environments. The results suggest that piperamides could be considered as potential AF agents.

Acknowledgements

This study was supported by a grant (DY125-15-T02) from the China Ocean Mineral Resources Research and Development Association, an award (SA-C0040/UK-C0016) from the King Abdullah University of Science and Technology to Pei-Yuan Qian and the National Natural Science Foundation of China (No. 21262047). The authors are grateful to the School of Chemistry and Biotechnology, Yunnan University of Nationalities for measuring the NMR and MS spectra. They also acknowledge Dr Zilong Wen of the Hong Kong University of Science and Technology for providing zebra iSh embryos.

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