A recent global ban on the use of organotin compounds as antifouling agents has increased the need for safe and effective antifouling compounds. In this study, a series of new butenolide derivatives with various amine side chains was synthesized and evaluated for their anti-larval settlement activities in the barnacle, Balanus amphitrite. Side chain modification of butenolide resulted in butenolides 3c-3d, which possessed desirable physico-chemical properties and demonstrated highly effective non-toxic anti-larval settlement efficacy. A structure-activity relationship analysis revealed that varying the alkyl side chain had a notable effect on anti-larval settlement activity and that seven to eight carbon alkyl side chains with a tert-butyloxycarbonyl (Boc) substituent on an amine terminal were optimal in terms of bioactivity. Analysis of the physico-chemical profile of butenolide analogues indicated that lipophilicity is a very important physico-chemical parameter contributing to bioactivity.

Keywords: antifouling; anti-larval settlement; barnacle; butenolides; structural optimization; side chain modification; lipophilicity

Introduction

Marine biofouling, the undesirable accumulation of microorganisms, algae and animals on submerged substrata, is a major problem for all marine industries. The attachment and growth of fouling organisms on manmade surfaces submerged in seawater often cause technical and economic problems (Richmond and Seed 1991; Townsin 2003; Schultz et al. 2011). Although antifouling (AF) paints containing organotins, lead, mercury or arsenic have been widely used to control biofouling in the past and have proven very effective, they are highly toxic and persistent once introduced into the marine environment (Voulvoulis et al. 2002; Konstantinou and Albanis 2004; Zhou et al. 2006). Given the International Maritime Organization (IMO) prohibition on the application of organotins to ships, effective from 17 September 2008 (Champ 2000), alternative AF paints with high levels of copper and herbicides have been used in recent years. However, these have also proven threatening to the marine environment because they can accumulate to high levels and have toxic effects on marine organisms (Omae 2003; Konstantinou and Albanis 2004; Bellas 2006; Thomas and Brooks 2010). Hence, there is an urgent need to develop new, effective, environmentally benign antifoulants.

One of the most ecologically relevant AF strategies formulated in response to this need has been to develop products based on the natural chemical defenses of sessile marine organisms that keep their body surfaces free of fouling (Clare 1996; Kitano et al. 2003; Nogata et al. 2003; Dobretsov et al. 2006; Fusetani 2011). Although more than 400 natural AF chemical compounds have been found to date, none have been developed into commercial antifoulants for two main reasons. Firstly, their yield tends to be poor and secondly, they are too structurally complex to be synthesized (Fusetani 2004, 2011; Qian et al. 2010a). The supply problem is a major challenge facing researchers aspiring to develop natural antifoulants. Some notable solutions to the supply issue have recently been put forward through the chemical synthesis of a number of AF candidates, such as the structural optimization of 3-alkylpridine and alkyl isocyanides (Kitano et al. 2003, 2011; Blihoghe et al. 2011). In previous studies conducted by the present authors, an analysis of the structure-activity relationships (SAR) of compounds isolated from marine organisms revealed that both furan and furanone
moieties were important functional pharmacophores for anti-larval settlement activity (Xu et al. 2010; Li et al. Forthcoming 2012). In this study, rather than investing more effort in searching for additional bioactive compounds, the authors aimed to optimize the structure of AF compounds based on SAR to develop new and effective antifoulants.

The recently developed compound 5-octylfuran-2(5H)-one (butenolide) (Xu et al. 2010) is a promising AF agent with great market potential due to its simple structure, strong AF effect and low toxicity. An initial study of the structure–activity relationships of alkyl butenolides isolated from the deep-sea actinomycete Streptomyces sp. showed that the 2-furanone ring was an essential element for bioactivity (Xu et al. 2010). Recent comparative proteomics and phosphoproteomics studies on the barnacle B. amphitrite and the bryozoan B. neritina have indicated that both of these species respond to butenolide by modulating their energy- and stress-related proteins (Qian et al. 2010b; Zhang et al. 2010). The species-specific morphological changes observed in B. amphitrite and B. neritina support the argument that butenolide has a species-specific AF mechanism (Zhang et al. 2011). A subsequent study based on an affinity pull-down assay has shown that B. amphitrite ACAT1 and B. neritina ACADVL, which are involved in the primary metabolism for energy production, are 2-furanone ring binding proteins (Zhang et al. 2012).

In addition to an ability to form a complex with a target protein, the effectiveness of a compound also generally depends on its ability to permeate cells and modulate the cellular signaling pathway (Camp et al. 2012). It is apparent from drug research that drug-like properties such as ClogP, which are often used to calculate lipophilicity and aqueous solubility, have a strong influence on cell permeability and the aqueous bioavailability of the drug (Kubinyi et al. 1979; Lo 2003). N-acyl is an essential functional group for many bioactive products such as N-acyl homoserine lactone (AHL) (Eberhard et al. 1981; Wilkinson et al. 2002; Nantasenamat et al. 2008) and has been used to optimize the lipophilicity and aqueous solubility of drugs (Kahns and Bundgaard 1991). A structure–activity relationship study of the AF amide analogues of loperamide suggested that amide moieties are important pharmacophores for potential AF activity (Moore et al. 2009). The aim of the present study was to shift the physico-chemical properties towards drug-like properties by modifying the side chain of alkyl butenolide, thereby developing new and effective AF candidates. First, the introduction of various N-acyl groups into the alkyl side chain led to the selection of a series of compounds containing a Boc terminal group within the side chain. Variation of the length of the alkyl side chain at the 5-position then improved lipophilicity through a desirable arrangement that led to the development of new, effective anti-larval settlement candidates 3c and 3d with EC50 values of 2.13 ± 0.54 and 2.22 ± 0.73 μM against B. amphitrite, respectively. This study also further explored the side chain SAR of butenolides and defined the correlation between physico-chemical properties and anti-larval settlement efficacy against B. amphitrite.

Materials and methods

Chemical synthesis of butenolide derivatives

All the chemical reagents employed in this study were purchased from Sigma Aldrich and Alfa Aesar. A simple solution adopted to regulate the ClogP of butenolide was the incorporation of an amine group into this side chain to allow for modification of the amine group. Incorporation of the amine side chain into the furan ring was initially targeted before investigating modification of the amine group and elongation of the carbon side chain (Figure 1) in order to explore the SAR of the side chain in more detail. The methods used to synthesize these compounds are detailed in Schemes 1–4 in Supplementary information [Supplementary information is available via a multimedia link on the online article webpage] (see also Blakemore et al. 1998, 1999; Molander and St Jean

Figure 1. Design and overall strategy for synthesis of butenolides.
Based on this approach, N-Boc protecting amine alkyl iodides 2a-c were reacted with 2-trimethylsiloxyfuran in the presence of silver trifluoroacetate to give rise to compounds 3a-c (Scheme S1, Table S2 in Supplementary information) (Jefford et al. 1988). Removal of the N-Boc protecting group from compounds 3a-f yielded compounds 4a-f. The presence of various acyl groups at the amine terminal of the side chain was also investigated. The reaction of compounds 4a and 4b with an excess of the appropriate carboxylic acid resulted in amides 5a-10a and 5b-10b (Table S1 in Supplementary information). All the target compounds (Figure 2) were subjected to $^1$H and $^{13}$C NMR and HRESI-MS analysis (NMR and MS data are shown in Supplementary information) to determine the criteria for their chemical structure or purity (> 95%) before biological testing.

**Anti-larval settlement assay**

Adults of *Balanus amphitrite* were collected from Pak Sha Wan, Hong Kong (22°19’N, 114°16’E) while adults of *Bugula neritina* were collected from submerged rafts at fish farms in Yung Shue O, Hong Kong (22°24’N, 114°21’E). Larvae were collected and cultured according to the method described by Dobretsov et al. (2007). Fresh competent larvae (capable of attachment and metamorphosis) were used in the bioassay. Anti-larval attachment activities were evaluated using cyprid larvae of *B. amphitrite* and swimming larvae of *B. neritina*. Larval settlement assays were performed using 24-well polystyrene plates (Becton Dickinson 353047). Each compound was first dissolved in a small amount of dimethyl sulfoxide (DMSO) at a concentration of 25.0 mg ml$^{-1}$ before being diluted with 0.22 µM of filtered seawater (FSW) to achieve a final concentration of 25.0 µg ml$^{-1}$ for the preliminary anti-larval settlement activity test. The active compounds were diluted with FSW to 50.0, 25.0, 10.0, 5.0, 2.0, 1.0, 0.5, 0.2 and 0.1 µg ml$^{-1}$ for further bioassay. About 15–20 competent larvae were gently transferred into each well with 1 ml of the testing solution while wells containing larvae in FSW with 0.1% DMSO served as the negative control. There were three replicates for each test solution. The plates for *B. amphitrite* were incubated for 48 h at 25°C while those for *B. neritina* were incubated for 24 h at 25°C. The effects of the compounds on larval settlement were determined by observing the plates under a dissecting microscope to check for (1) settled larvae, (2) swimming larvae, and (3) dead larvae. The number of settled larvae was expressed as a percentage of the total number of larvae per well.

To calculate the EC$_{50}$ and LC$_{50}$ values of each compound, a concentration–response curve was initially plotted, followed by the construction of a trend line for each compound. EC$_{50}$ was calculated as the concentration at which 50% of the larval population was inhibited from settling, compared with that in the negative control while LC$_{50}$ was calculated as the concentration at which 50% of the larval population died. Each compound was tested using three different
batches of larvae to determine the mean and standard deviation (SD) of the EC$_{50}$ and LC$_{50}$ values.

**Results and discussion**

**Anti-larval settlement activity against B. amphitrite**

**Impact of the nature of the side chain on anti-larval settlement activity**

Barnacles are among the most predominant marine fouling organisms and their hard shells make it extremely difficult to remove them from marine installations (Khandeparker and Anil 2007). The barnacle *B. amphitrite* is one of the primary model organisms used extensively in screening for AF substances. To explore the SAR of butenolides with modified side chains, anti-larval settlement activity in two series of analogues containing modified side chains was evaluated through an anti-larval settlement bioassay. These two series of analogues were 2-furanone derivatives with a 5 or 6 carbon alkyl amine substituent at the 5-position. The results show that the Boc carbamate analogues (3a and 3b) exhibited the best anti-larval settlement efficacy with EC$_{50}$ values of 5.43 ± 0.67 and 4.00 ± 0.85 µM, respectively (Table 1, Figure 3). SAR analysis indicates that the Boc group was the optimal terminal group as a substituent at the N-terminal, with Boc (3a and 3b, EC$_{50}$ = 5.43 and 4.00 µM) > COCF$_3$ (7a and 7b, EC$_{50}$ = 9.00 and 7.80 µM) > COCH$_3$ (6a and 6b, EC$_{50}$ = 16.8 and 18.0 µM) > CHO (5a and 5b, EC$_{50}$ = 40.3 and 26.3 µM) > H (4a and 4b, EC$_{50}$ = 444 and 225 µM) in terms of anti-larval settlement activity. Increases in bioactivity appear to be related to the lipophilicity of the substituents. The bioactivity of compounds 8a-b was comparable to that of 3a-b, respectively, whereas the bioactivity in 9a-b and 10a-b was significantly lower. The anti-larval settlement activity for aromatic analogues 8-10a and 8-10b was not enhanced compared with that in Boc analogues. Increases in bioactivity in C5 and C6 alkyl side chain analogues (3-5a, 3-5b) appear to be related to the length of their alkyl side chains, which also affected compound lipophilicity. Furthermore, a high degree of hydrophilic substitution in the side chain resulted in a loss of bioactivity, as exemplified by 4a and 4b, which further suggests that the lipophilicity of the side chain might be a key factor in compound bioactivity, which is consistent with a previous study (Xu et al. 2010).

**Impact of the length of the carbon side chain on anti-larval settlement activity**

As seen in previous side chain variations, the Boc group was the optimal terminal group. Variation in the length of the alkyl amine side chain from 6 carbons to 10 carbons resulted in a series of carbamate analogues within the Boc group and their amine and formic amide analogues (see Supplementary information). [Supplementary information is available via a multimedia link on the online article webpage.] Table 2 provides the anti-larval settlement data for all of these

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**Table 1. Anti-larval settlement activities of butenolide analogues (3-10a and 3-10b) against the barnacle *B. amphitrite*.**

| R | Compound | EC$_{50}$ (µM) | LC$_{50}$/EC$_{50}$ | Compound | EC$_{50}$ (µM) | LC$_{50}$/EC$_{50}$ |
|---|----------|----------------|---------------------|----------|----------------|---------------------|
| 3 | Boc      | 3a             | 5.43 ± 0.67         | >35      | 3b             | 4.00 ± 0.85         |
| 4 | H        | 4a             | 444 ± 17            | UD       | 4b             | 225 ± 31            |
| 5 | CHO      | 5a             | 40.3 ± 5.7          | >6       | 5b             | 26.3 ± 6.2          |
| 6 | COCH$_3$ | 6a             | 16.8 ± 3.1          | >14      | 6b             | 18.0 ± 5.7          |
| 7 | COCF$_3$ | 7a             | 9.00 ± 1.78         | >21      | 7b             | 7.80 ± 1.82         |
| 8 |           | 8a             | 4.07 ± 0.69         | >41      | 8b             | 6.06 ± 0.59         |
| 9 |           | 9a             | 12.4 ± 2.0          | >15      | 9b             | 14.4 ± 2.53         |
| 10|          | 10a            | 9.56 ± 1.41         | >17      | 10b            | 6.43 ± 1.22         |

![Figure 3. Anti-larval settlement activities of butenolide analogues 3-10a and 3-10b against the barnacle *B. amphitrite.*](image-url)
analogues. As seen in the variation of 4, an increase in the number of carbons in the side chain of an amine analogue from C5 to C10 significantly increased the anti-larval settlement activity (all \( p < 0.05 \)) (Figure 4). Similar tendencies were also exhibited in amide analogues (5a-f), which suggests that the enhancement of larval settlement inhibition is correlated with an increase in the number of carbons in the alkyl side chain. With respect to the variation of compound 3, increases in the anti-larval settlement activity of compounds 3a-c depended on the number of carbons in the alkyl side chain moiety (Figure 5). The bioactivity of compounds 3d-e was comparable to that of compound 3c, whereas compound 3f was significantly less effective (\( p < 0.05 \)). No enhancement in anti-larval settlement efficacy was observed when the number of carbons in the alkyl chain side was increased beyond C7. Compared with the positive control 5-octylfuran–2(5H)-one (butenolide) and Sea-nine 211, compounds 3d-e were all exhibited significantly greater activity (\( p < 0.05 \)), with EC\(_{50}\) values of 2.13 ± 0.54, 2.22 ± 0.73 and 2.44 ± 0.78 \( \mu \)M, respectively. Compounds 3a-b, with EC\(_{50}\) values of 5.43 ± 0.67 and 4.00 ± 0.85 \( \mu \)M, respectively, were comparable to 5-octylfuran-2(5H)-one (butenolide), but significantly less effective than compounds 3c-e (\( p < 0.05 \)). Therefore, varying the length of the alkyl side chain had a notable effect on the biological activity of these compounds, with an alkyl side chain comprising seven to eight carbons being optimal.

### Table 2. Anti-larval settlement activities of butenolide analogues (3a-f, 4a-f and 5a-f) against the barnacle *B. amphitrite*.

| Side chain | Compound | EC\(_{50}\) (\( \mu \)M) | LC\(_{50}/EC_{50}\) | Compound | EC\(_{50}\) (\( \mu \)M) | LC\(_{50}/EC_{50}\) | Compound | EC\(_{50}\) (\( \mu \)M) | LC\(_{50}/EC_{50}\) |
|------------|----------|--------------------------|------------------|----------|--------------------------|------------------|----------|--------------------------|------------------|
| n = 1      | 3a       | 5.43 ± 0.67              | >35              | 4a       | 444 ± 17                 | UD               | 5a       | 40.3 ± 5.7               | 6                |
| n = 2      | 3b       | 4.00 ± 0.85              | >46              | 4b       | 225 ± 32                 | UD               | 5b       | 26.3 ± 6.2               | 9                |
| n = 3      | 3c       | 2.13 ± 0.54              | >61              | 4c       | 108 ± 15                 | UD               | 5c       | 23.0 ± 1.9               | 10               |
| n = 4      | 3d       | 2.22 ± 0.73              | >73              | 4d       | 36.1 ± 4.2               | 7                | 5d       | 14.3 ± 1.9               | 15               |
| n = 5      | 3e       | 2.44 ± 0.78              | >63              | 4e       | 22.0 ± 1.9               | 10               | 5e       | 7.50 ± 0.16              | 26               |
| n = 6      | 3f       | 4.57 ± 1.08              | >32              | 4f       | 16.7 ± 2.5               | 13               | 5f       | 5.48 ± 0.81              | 30               |

| Butenolide | 5.96 ± 1.56 | >43 | Sea-nine211 | 5.76 ± 1.05 | |

### Table 3. Anti-larval settlement activities of compounds synthesized against the bryozoan *B. neritina*.

| Compound | EC\(_{50}\) (\( \mu \)M) | LC\(_{50}/EC_{50}\) | Compound | EC\(_{50}\) (\( \mu \)M) | LC\(_{50}/EC_{50}\) |
|----------|--------------------------|------------------|----------|--------------------------|------------------|
| 3b       | 76.6 ± 13.2              | >5               | 5e       | 47.0 ± 10.4              | >4               |
| 3c       | 10.1 ± 4.0               | >20              | 7f       | 21.5 ± 1.2               | >10              |
| 3d       | 9.8 ± 2.4                | >35              | 7b       | 88.4 ± 10.0              | >6               |
| 3e       | 6.21 ± 1.58              | >39              | 8b       | 27.4 ± 5.3               | >7               |
| 3f       | 9.71 ± 1.30              | >26              | 10b      | 36.8 ± 11.5              | >17              |

Figure 4. Anti-larval settlement activities of compounds 4a-f and 5a-f against the barnacle *B. amphitrite*.

Figure 5. Anti-larval settlement activities of synthesized compounds 3a-f and positive control group 5-octylfuran-2(5H)-one (A) and Sea-nine 211 (B) against the barnacle *B. amphitrite*. The histogram data are expressed as means.
Impact of physico-chemical properties on anti-settlement activity

Lipinski’s seminal investigation leading to the ‘rule of five’ has become one of the tools most widely used to assess the relationships between structures and drug-like properties (Lipinski et al. 1997). Drug-like properties such as median ClogP and aqueous solubility have a substantial influence on the ADME (absorption, distribution, metabolism and excretion) properties of drugs and the environmental fate of chemicals, such as their uptake, bioaccumulation and sediment-water partition coefficients (Kubinyi et al. 1979; Cronin et al. 2006; Leeson and Springthorpe 2007). In addition to the notable effect that varying the side chain has on biological activity, the side chain SAR described above also reveals a potential correlation between lipophilicity and biological activity. Given the variety of butenolide analogues at hand, the authors conducted a Pearson correlation analysis (SPSS 16.0) to investigate the relative impact of physico-chemical parameters (Table S3 in Supplementary information) [Supplementary information is available via a multimedia link on the online article webpage] on anti-larval settlement efficacy (Table 4). Molecular weight (MW) and lipophilicity, as calculated by ClogP, were negatively correlated with the natural logarithm of the EC_{50} value (LnEC_{50}), whereas the latter had a positive correlation with aqueous solubility (ALOGpS). Individual correlation analysis showed a significant negative correlation between ClogP and LnEC_{50} (R = -0.921, p < 0.001; Figure 6). These results indicate that the higher the degree of lipophilicity, the lower the EC_{50} concentration. However, lipophilicity had a limited positive effect on anti-larval settlement efficacy (LogP < 5.0) in accordance with Lipinski’s ‘rule of five’. A similar negative correlation between MW and LnEC_{50} (R = -0.871, p < 0.001; Figure 7) might be a result of the positive association between MW and lipophilicity (ClogP) (R = -0.914, p < 0.001). In this study, modified target compounds 3c and 3d within a median ClogP and MW arrangement complied with Lipinski’s ‘rule of five’ and showed the desirable physico-chemical space considered essential for aqueous bioavailability/cell permeability. These data indicate that lipophilicity is the most important parameter contributing to the activity of butenolide analogues. One of the other key parameters observed here was aqueous solubility.

Table 4. Correlation coefficients (below diagonal) and uncorrected P-values (above diagonal) between bioactivity (Ln (EC_{50})) and physico-chemical parameters (MW, CLogP, ALOGpS, HBA, HBD and RingAr).

|         | Ln(EC_{50}) | MW  | CLogP       | ALOGpS | HBA | HBD | RingAr |
|---------|-------------|-----|-------------|--------|-----|-----|--------|
| Ln(EC_{50}) | <0.001*     |     | <0.001*     | <0.001*| 0.0298 | 0.115 |
| MW      | -0.871*     |     | <0.001*     | <0.001*| 0.022 | 0.004 |
| CLogP   | -0.921*     | 0.914* |         | <0.001*| 0.237 | 0.361 |
| ALOGpS  | 0.791*      | -0.895* | -0.791*  | <0.001*| 0.024 | 0.096 | 0.111 |
| HBA     | -0.569      | 0.636* | 0.668*     | -0.377 | 0.003 |
| HBD     | 0.105       | -0.383 | -0.141     | 0.254  | -0.501 | 0.003 |
| RingAr  | -0.234      | 0.493 | 0.361       | -0.43  | 0.65* |

*show significant correlation coefficients and P-values after Bonferroni correction; ^physico-chemical parameters detailed in Supplementary information.
**Anti-larval settlement activity against the bryozoan**

*Bugula neritina*

The bryozoan *B. neritina* is a common and highly abundant soft fouling organism found worldwide. The animals tolerate high levels of pollution, including copper, which makes it difficult for copper-based AF paints to control them (Piola and Johnston 2006). To explore the AF potency of the butenolide derivatives synthesized in this study in more detail, the ability of all of the derivatives to inhibit larval settlement was investigated. The results (Table 3) show that a 9-carbon alkyl side chain with a Boc substituent on the terminal remained optimal, as exemplified by compound 3c (7.21 ± 0.58 μM). This further supports the argument that the side chain of a compound has a profound effect on its bioactivity.

**Conclusion**

This study analyzed the synthesis of butenolide analogues with a potent ability to inhibit anti-larval settlement of both *B. amphitrite* and *B. neritina*. Representative analogue butenolides 3c and 3d with desirable physico-chemical properties (median ClogP and MW) were found to be the most potent antifoulants, with AF activity EC50 values of 2.13 ± 0.54 and 2.22 ± 0.73 μM against *B. amphitrite*, respectively. An analysis of structure–activity relationships revealed that the anti-larval settlement efficacy differed significantly according to the structural properties of the side chain. The positive association between lipophilicity and bioactivity was also illustrated using a Pearson correlation analysis. The results of this study indicate that researchers should consider modifying the side chains and the lipophilicity of bioactive compounds when developing effective antifoulants.

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