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ORIGINAL ARTICLE

Antifouling Activity of Secondary Metabolites Isolated from Chinese Marine Organisms

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Abstract Biofouling results in tremendous economic losses to maritime industries around the world. A recent global ban on the use of organotin compounds as antifouling agents has further raised demand for safe and effective antifouling compounds. In this study, 49 secondary metabolites, including diterpenoids, steroids, and polyketides, were isolated from soft corals, gorgonians, brown algae, and fungi collected along the coast of China, and their antifouling activity was tested against cyprids of the barnacle *Balanus* (*Amphibalanus*) *amphitrite*. Twenty of the compounds were found to inhibit larval settlement significantly at a concentration of 25 μg ml^{-1}. Two briarane diterpenoids, juncin O (**2**) and juncenolide H (**3**), were the most promising non-toxic antilarval settlement candidates, with EC_{50} values less than 0.13 μg ml^{-1} and a safety ratio (LC_{50}/EC_{50}) higher than 400. A preliminary structure—activity relationships study indicated that both furanon and furan moieties are important for antifouling activity. Intriguingly, the presence of hydroxyls enhanced their antisettlement activity.

Keywords Antifouling · Antilarval settlement · Structure—activity relationship · Marine natural products · *Balanus amphitrite*

Introduction

Marine biofouling refers to the undesirable accumulation of microorganisms, algae, and animals on submerged substrates, leading to subsequent biodeterioration. The attachment and growth of such fouling organisms as barnacles, hydroids, and mussels on man-made surfaces submerged in seawater result in a number of technical and economic problems (Richmond and Seed 1991; Townsin 2003). Antifouling (AF) paints have been used to combat these problems and to protect ship hulls, aquaculture cages, and other marine installations. Paints containing organotins, copper, lead, mercury, or arsenic, which were widely used to control biofouling in the past, are very effective, but also highly toxic and persistent in the marine environment (Voulvoulis et al. 2002; Konstantinou and Albanis 2004; Zhou et al. 2006). A total ban on the production of tributyltin (TBT)-based coatings was implemented in January 2003, and the International Maritime Organization (IMO) has prohibited their application to ships since September 17, 2008 (Sonak et al. 2009). Alternative AF paints that contain high levels of copper and biocides, such as Irgarol 1051, dichlofluanid, diuron, and chlorothalonil, have been used in recent years (Omae 2003), but unfortunately, these have also proved to pose a threat to the marine environment, as they can accumulate to high levels in coastal waters and contaminate the food chain (Konstantinou and Albanis 2004; Bellas

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2006; Thomas and Brooks 2010). Copper- and other biocide-based marine coatings must eventually be replaced by new, effective, and environmentally benign AF compounds.

In response to the urgent demand for such compounds, considerable efforts have been made in recent years to find efficient and environmentally friendly AF agents and technologies (Dobretsov et al. 2006). One of the most ecologically relevant AF strategies is the development of products that are based on the natural chemical defenses of sessile marine organisms such as coral and seaweed, thus keeping their body surfaces free of fouling (Wahl 1989; Clare 1996; Rittschof 2000; Nogata 2003; Fusetani 2004). Marine natural products capable of inhibiting one or several stages of fouling on ship hulls and other submerged structures may be potential sources of environmentally compatible, non-toxic, or low-toxicity antifoulants (Sears et al. 1990; Egan et al. 2001; Hattori et al. 2001; Bhadury and Wright 2004; Fusetani 2004; Chambers et al. 2006; Li et al. 2006; Fusetani and Clare 2006; Barbosa et al. 2007; Hellio and Yebra 2009). A number of recent reviews highlight the achievements of marine natural products in affording AF candidates, with approximately 50 terpenoids and 10 steroids identified as potential candidates to date, although no obvious structure—activity relationship has been recognized (Fusetani 2004, 2011; Qian 2010). Accordingly, we have recently initiated a program to discover AF natural products from marine organisms, and here, we discuss their structure—activity relationships.

Barnacles are among the most predominant fouling organisms, and their hard shells make them extremely difficult to remove from ship hulls (Khandeparker and Anil 2007). They are thus the primary model organisms used in the search for AF substances. In this study, we first tested the antilarval settlement activities against *Balanus amphitrite* of 49 compounds extracted and purified from fungi, algae, and corals collected in coastal areas of China. The primary structure—activity relationships among the compounds were also investigated.

Materials and Methods

Marine Organisms

The corals used in this study were collected from a coral reef off Weizhou Island and from the Sanya Meishan and Lingchang reefs in the South China Sea. The algae were collected from Sanya Lingshuiwan, also in the South China Sea. Both the corals and algae were identified by Professor H. Huang of the South China Sea Institute of Oceanology at the Chinese Academy of Sciences. The strains of the genera *Aspergillus* and *Alternaria* were isolated from a piece of tissue in the inner part of the *corals* and identified according to their morphological characteristics and a molecular biological protocol involving 16 s RNA amplification and sequencing of the internal transcribed spacer region, as described in the literature (Zheng et al. 2012). Extraction and purification of the AF compounds and their structural determination were carried out as described in the Supporting Information.

Larval Culture of the Barnacle *B. amphitrite*

Adults of the barnacle *B. amphitrite* Darwin that had been exposed to air for more than 6 h were collected from the intertidal zone of Hong Kong (22°19′ N, 114°16′ E) and then placed in a container filled with 0.22 μm of fresh filtered seawater (FSW) to release the nauplii following the protocol described in the literature (Harder et al. 2001). The newly released nauplii were then transferred with a pipette to clean culture beakers containing FSW and reared to the cyprid stage following the method described by Thiyagarajan et al. (2003). The larvae were kept at 26–28 °C and fed with *Chaetoceros gracilis*, and they developed into cyprids on the fourth day. Fresh cyprids (0–4 h) were used in the bioassay.

Antifouling Bioassay

The test compounds were first dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted with filtered FSW to achieve final concentrations of 25 and 50 μg ml^{-1} for the preliminary testing of their AF effects. The active compounds were then diluted to 0.13, 0.63, 1.25, 2.50, 5.00, 10.0, and 20.0 μg ml^{-1} for further bioassay. Fifteen to 20 competent larvae (Head et al. 2003) were gently transferred into each well with 1 ml of testing solution in three replicates, and the wells containing larvae in FSW with DMSO alone served as the controls. The plates were incubated for 24–48 h at 23 °C. The compounds' effects on larval settlement were determined by examining the plates under a dissecting microscope to check for (1) settled larvae, (2) non-settled larvae, and (3) any possible toxic effects, such as death or paralysis. The number of settled or metamorphosed larvae was expressed as a percentage of the total number of larvae added to each well.

The EC_{50} (the minimum concentration that inhibited 50 % of cyprid settlement compared with the negative control) and LC_{50} (the lethal dose that killed 50 % of the cyprids compared with the negative control) (Rittschof 2001) values were calculated using the Probit software program. For the calculation of the EC_{50} and LC_{50} values of the compounds, a concentration–response curve was plotted, and a trend line was then constructed for each compound.

Results and Discussion

Isolated Compounds

Structural determination of the 49 compounds, e.g., 23 diterpenoids (**1**–**23**), 18 steroids (**24**–**41**), and 8 polyketides (**42**–**49**), was carried out as described in the Supporting Information (see the Structure determination section and Figs. 1–3 of this information). Most of the briaranes (**1**–**10**), including juncin O-P (**2**, **4**) (Qi et al. 2004), juncenolide H (**3**) (Wang et al. 2009), and juncin Z1 (**5**) (Qi et al. 2006), were characteristic secondary metabolites of gorgonian of the genus *Dichotella*. Eleven cembranoids (**13**–**23**), including sarcolactone A (**13**) (Sun et al. 2010) and sarcophytonolide H (**14**) (Jia et al. 2006), were isolated from the soft coral of the genus *Sarcophyton*. The sources of sterols were diverse, including coral, algae, and fungus. A number of polyketides (**42**–**49**), including sterigmatocystin (**42**) and methoxysterigmatocystin (**43**) (Holker and Kagal 1968), were isolated primarily from marine-derived fungi of the genera *Aspergillus* and *Alternaria.*

Screening for Compounds with Antilarval Settlement Activity

To establish the baseline antilarval settlement potency of the marine natural products isolated, we first tested 23 diterpenoids (**1**–**23**), 18 steroids (**24**–**41**), and 8 polyketides (**42**–**49**) for their ability to inhibit the larval settlement of *B. amphitrite* at concentrations of 50 and 25 μg ml^{-1}. The 20 compounds shown in Table 1 were found to completely inhibit larval settlement at 50 μg ml^{-1}, in which 18 inhibit settlement at 25 μg ml^{-1}. This concentration was established as the efficacy level for natural AF agents in a US Navy program (Kwong et al., 2006).

Detection of EC_{50} and LC_{50}/EC_{50} values

The LC_{50}/EC_{50} ratio, which is often referred to as the therapeutic ratio, is commonly used as a yardstick for a compound's potential (Fusetani et al. 2011; Qian et al. 2010). Only compounds with a therapeutic LC_{50}/EC_{50} ratio>50 and EC_{50}<5.0 μg ml^{-1} are considered to be promising non-toxic AF candidates (Qian et al. 2010). The compounds that proved effective at a concentration of 25 μg ml^{-1} were further tested using the same bioassay procedure as that in the preliminary screening to detect their EC_{50} and LC_{50} values. Table 1 shows the antilarval settlement activity of these compounds against *B. amphitrite.* Eleven compounds (**1**–**5**, **10**–**12**, **26**, and **42**–**43**) demonstrated potent activity with EC_{50} values lower than or comparable to 1.0 μg ml^{-1}, whereas the other nine exhibited moderate activity ranging from 5.13 to 16.7 μg ml^{-1}. Among the 10 potent active compounds, compounds **42** and **43**, which were isolated from fungus *Aspergillus* sp., showed paralyzing effects on the barnacle larvae. The other eight had no recordable toxic effect, even at 50 μg ml^{-1}. The LC_{50}/EC_{50} ratios of these eight compounds were higher than or comparable to 50, suggesting that they are either weakly toxic or completely non-toxic AF compounds that inhibit the larval settlement. Of the potent antilarval settlement compounds, three briaranes isolated from gorgonian *Dichotella gemmacea*, namely, juncin O (**2**), juncenolide H (**3**), and juncin Z1 (**5**), proved to be the most potent non-toxic antilarval settlement candidates, as their EC_{50} values were less than 0.13 μg ml^{-1}, which is 10 times lower than that of SeaNine 211 (Jacobson and Willingham 2000), and their LC_{50}/EC_{50} ratios were higher than 400. One steroid, suberoretisteroid A (**26**), which was isolated from the gorgonian *D. gemmacea*, also displayed potent activity, with an EC_{50} value of 0.81 μg ml^{-1} and LC_{50}/EC_{50} ratios higher than 61.7.

Structure—Activity Relationships of Larval Settlement Inhibitors

A preliminary structure—activity relationship investigation suggested that the AF functional group was the furan ring in briarane-type diterpenoids (Clare 1999; Qi et al. 2006). In an earlier work, we also suggested that the 2-furanone ring is the functional moiety responsible for AF activity in butenolides (Xu et al. 2010; Li et al. 2012). For comparison, we discuss here the structure—activity relationships of the 49 marine natural products isolated in the current study. As shown in Fig. 1 and Table 1, compounds **1**–**5** and **8**–**14**, having furanone moiety, displayed significant AF activity and EC_{50} values less than 10.0 μg ml^{-1}, whereas the cembranoids (compounds **16**–**19** and **21**–**23**) with no furanone moiety exhibited no such activity. These results further support our earlier conclusion that furanone moiety is an important pharmacophore in the inhibition of *B. amphitrite* cyprid settlement. Compounds **13** and **14** exhibited mild AF activity, with EC_{50} values of 6.27 and 5.98 μg ml^{-1}, respectively, but their analogues (compounds **21** and **18**), which lack furanone moiety, did not inhibit the larval settlement of barnacle cyprids at a concentration of 25 μg ml^{-1}.

The presence of furan moiety in other compound skeletons was also found to influence AF efficacy. As previously noted, of the 18 steroids (compounds **24**–**41**), only compounds **24**–**26**, which possess furan moiety in the side chain, exhibited AF activity, with EC_{50} values of 7.31, 7.91, and 0.81 μg ml^{-1}, respectively (Kong et al. 2012). Compounds **27**–**41**, in contrast, in which furan moiety is not present, displayed no inhibitory effect on the larval

Table 1 Antifouling activity and therapeutic ratio of 49 compounds isolated in this study

Compound	Resource	EC_{50} (μg ml^{-1})	LC_{50}/EC_{50}
12-Epi-fragilide G (**1**)	*Dichotella gemmacea*	1.18	>42.3
Juncin O (**2**)	*D. gemmacea*	<0.125	>400
Juncenolide H (**3**)	*D. gemmacea*	<0.125	>400
Juncin P (**4**)	*D. gemmacea*	0.77	>64.9
Juncin Z1 (**5**)	*D. gemmacea*	<0.125	>400
Praelolide (**6**)	*D. gemmacea*	>50	UD
Junceellin A (**7**)	*D. gemmacea*	>50	UD
Juncin U (**8**)	*D. gemmacea*	6.54	>7.64
Juncenolide D (**9**)	*D. gemmacea*	6.35	>7.87
Junceellolide D (**10**)	*D. gemmacea*	0.80	>62.5
Reticulolide (**11**)	*Subergorgia mollis*	0.35	>142
Robustolide A (**12**)	*S. mollis*	0.86	>58.1
Sarcolactone A (**13**)	*Sarcophyton infundibuliforme*	6.27	>7.97
Sarcophytonolide H (**14**)	*S. crassocaule*	5.98	>8.36
Sarcophytonolide J (**15**)	*S. crassocaule*	>25	UD
Cembrene C (**16**)	*S. infundibuliforme*	>25	UD
(1S)-isosarcophytol A (**17**)	*S. infundibuliforme*	>25	UD
(R)-sarcophytol A (**18**)	*S. crassocaule*	>25	UD
Sarcophytol B (**19**)	*S. crassocaule*	>25	UD
Marasol (**20**)	*S. crassocaule*	12.5	>4.00
Sarcophytol E (**21**)	*S. crassocaule*	>25	UD
(7*R*,8*R*,14*S*,1*E*,3*E*,11*E*)-7,8-epoxycembra-1,3,11-trien-14-ol (**22**)	*S. crassocaule*	>25	UD
Sarcophytol O (**23**)	*S. crassocaule*	>25	UD
22-Acetoxy-3,25-dihydroxy-16-24,20-24-bisepoxy-(3β,16α,20S,22R,24S)-cholest-5-ene (**24**)	*S. mollis*	7.31	>6.83
Suberoretisteroid C (**25**)	*D. gemmacea*	7.91	>6.32
Suberoretisteroid A (**26**)	*D. gemmacea*	0.81	>61.7
3β-cCholest-5-ene-3,16-diol(**27**)	*Kjellmaniella crassifolia*	>50	UD
Stigmasta-5,22-*E*-,28-triene-3β,24α-diol (**28**)	*Sargassum thunbergii*	>50	UD
Acanthovagasteroid D (**29**)	*Anthogorgia caerulea*	>50	UD
Provitamin D (**30**)	*Aspergillus* sp.	>50	UD
(22*E*)Cholesta-5,22-dien-3-one, (**31**)	*Scleronephthya* sp.	>50	UD
Pregna-1,4,20-trien-3-one (**32**)	*S. gracillimum*	>50	UD
Pregna-1,4-dien-3-one (**33**)	*S. gracillimum*	>50	UD
Dendronesterols A (**34**)	*Scleronephthya* sp.	>50	UD
Cholest-7-ene-3β,5α,6β-triol (**35**)	*Scleronephthya* sp.	>50	UD
(22Z, 24S)cerevisterol (**36**)	*Scleronephthya* sp.	>50	UD
Astrogorgiadiol (**37**)	*Muricella sibogae*	>50	UD
Calicoferol A (**38**)	*M. sibogae*	>25	UD
Calicoferol E (**39**)	*M. sibogae*	>25	UD
Peroxyergosterol (**40**)	*Aspergillus* sp.	>50	UD
Stigmasta-5,8-epidioxy −6,22-dien-3-ol (**41**)	*D. gemmacea*	>25	UD
Sterigmatocystin (**42**)	*Aspergillus* sp.	<0.125	paralysis
Methoxysterigmatocystin (**43**)	*Aspergillus* sp.	<0.125	paralysis
6,8-di-O-Me versiconol (**44**)	*Aspergillus* sp.	5.13	>9.74
Amibromdole (**45**)	*Alternaria sp.*	16.7	>2.99
Altersolanol L (**46**)	*Alternaria sp.*	>50	UD
Altersolanol C (**47**)	*Alternaria sp.*	>25	UD
Physcion (**48**)	*Alternaria sp.*	>50	UD
7-OH-2-(2-hydroxypropyl)-5-methyl-Benzopyran-4-one (**49**)	*Aspergillus* sp.	>50	UD
SeaNine 211	Positive control	1.23	20.3

UD undetectable

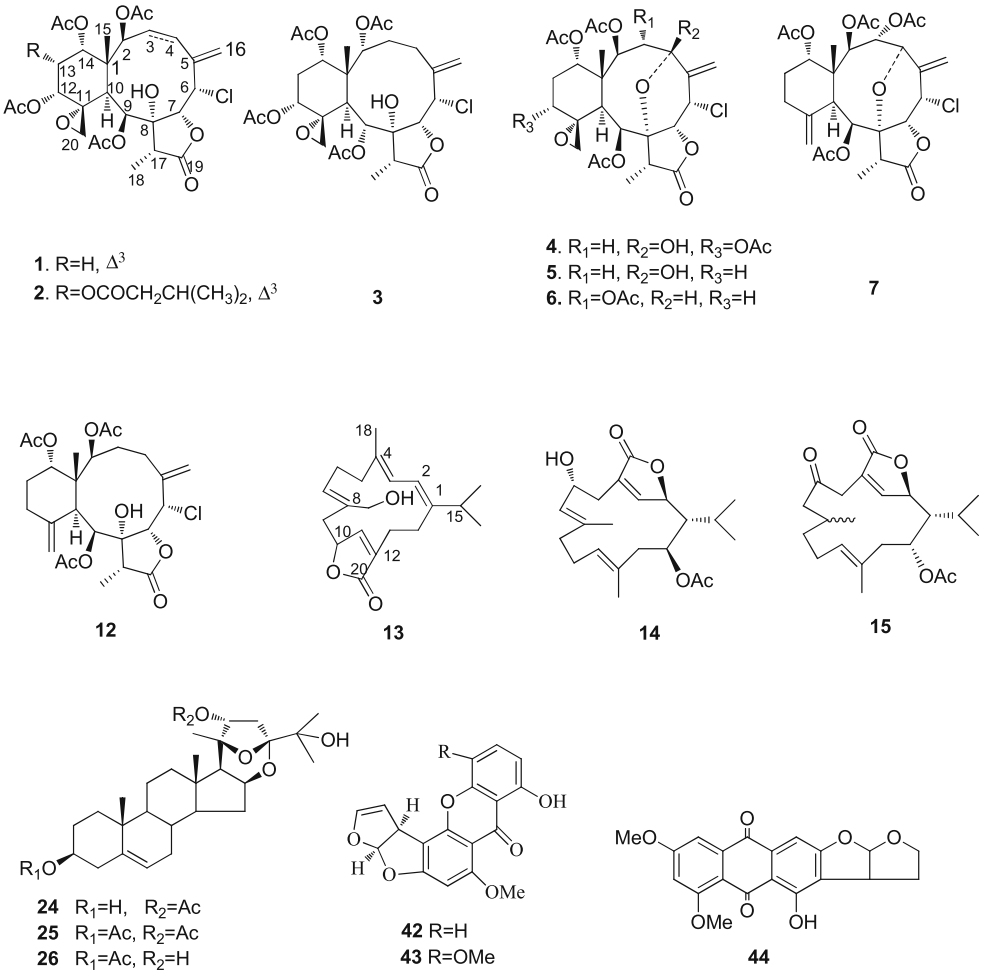

Fig. 1 Structures of the selected secondary metabolites isolated from marine resources

settlement of the barnacle *B. amphitrite*, even at a concentration of 50 μg ml^{-1}. The positive effect of furan moiety on antilarval settlement also appeared in the polyketides, as exemplified by compounds **42**–**44** (<0.13, <0.13, and 5.13 μg ml^{-1}). Compound **44**, which contains an additional tetrahydrofuran ring, exhibited 10-fold greater efficacy than **48**. Thus, the data obtained in this study using compounds of different skeletons provide further support for the essential role played by furan moiety in antilarval settlement efficacy.

Although most briaranes with a furan ring exhibited antisettlement activity against barnacle larvae, those without a hydroxyl group displayed no AF effect, as exemplified by compounds **6**, **7**, and **15**, thus suggesting that the presence of a hydroxyl functional group influences the antilarval settlement efficacy of natural compounds. Two briarane diterpenoids (compounds **4** and **5**) showed potential AF activity with EC_{50} values of 0.77 μg ml^{-1} and less than 0. 13 μg ml^{-1}, whereas their analogues without hydroxyl group at C-4, compounds **6** and **7**, failed completely to inhibit barnacle larval settlement. The presence of a hydroxyl group at C-8 was also found to influence compound bioactivity, as exemplified by **12** (0.86 μg ml^{-1}) in comparison with compounds **7** (>50.0 μg ml^{-1}). Similar tendencies were also found in the cembranoids and steroids. Compounds **13** and **14** exhibited moderate AF activity, with EC_{50} values of 6.27 and 5.98 μg ml^{-1}, respectively, whereas compound **15**, which lacks a hydroxyl group on C-6, exhibited no AF activity. Compounds **26** exhibited strong AF activity with EC_{50} values of 0.08 μg ml^{-1}, whereas compound **15**, which lacks a hydroxyl group on C-24, exhibited 10-fold lower AF activity (EC_{50} 7.91 g ml^{-1}).

Conclusion

Marine organisms have developed chemical defense systems that keep their body surfaces free of fouling, and their metabolites, which span a wide range of chemical classes (e.g., terpenoids, steroids, and polyketides), are an excellent source of non-toxic AF compounds. Of the active compounds identified in the study reported herein, seven diterpenoids and one steroid isolated from corals exhibited potent antilarval settlement activities and featured EC_{50} values less than 1.00 μg ml^{-1} and an LC_{50}/EC_{50} ratio higher than 50. Representative compounds juncin O (**2**) and juncenolide (**3**) were found to be the

most promising non-toxic antilarval settlement candidates. Our investigation of the structure—activity relationships among the 49 compounds of different skeletons revealed that furan and furanone moieties were important functional pharmacophores for potential AF activity in natural compounds. Intriguingly, the presence of hydroxyls in natural compounds with furan moieties was found to influence antilarval settlement efficacy.

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