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Short communication

Antifouling activity against bryozoan and barnacle by cembrane diterpenes from the soft coral *Sinularia flexibilis*

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ABSTRACT

In the present study, seven cembrane diterpenes were isolated from the soft coral *Sinularia flexibilis* by Toyopearl HW-40 column chromatography and High Performance Liquid Chromatography (HPLC). The diterpenes were identified as epoxycembrane A (**1**), sinularin (**2**), sinulariolide (**3**), (1*R*,13*S*,12*S*,9*S*,8*R*,5*S*,4*R*)-9-acetoxy-5,8:12,13-diepoxycembr-15(17)-en-16,4-olide (**4**), 11-dehydrosinulariolide (**5**), (−)14-deoxycrassin (**6**) and dihydrosinularin (**7**). The antifouling activity of these compounds was examined by settlement assays, using the larvae of the bryozoan *Bugula neritina* and the barnacle *Balanus albicostatus*. With the exception of compound **2**, all compounds indicated significant antifouling activity and a variety of EC_{50} values. In particular, compound **6** exhibited remarkable anti-settlement activity against the two biofoulers (EC_{50} for *B. neritina* 3.90 μg ml^{-1}; EC_{50} for *B. albicostatus* 21.26 μg ml^{-1}) as well as low toxicity against *B. albicostatus* larvae (LC_{50} > 100 μg ml^{-1}), suggesting its potential as an environmentally friendly antifoulant. This is the first report on the antifouling activity of compounds **1** and **4**–**7**, further demonstrating the involvement of cembrane diterpenes in the chemical defense of soft corals against surface fouling.

1. Introduction

Toxic antifoulants such as tributyltin and copper have been widely used on the surfaces of artificial structures submerged in the sea in order to deter marine fouling organisms (Yebra et al., 2004; Muynck et al., 2009; Dafforn et al., 2011). However, a growing awareness of their adverse environmental impacts has led to strict regulations on their use as marine antifoulants (Thomas and Brooks, 2010; Dafforn et al., 2011). This has triggered the search for environmentally friendly natural antifouling products. A great deal of effort has been directed towards the screening of natural product antifoulants (NPAs) from marine organisms, especially sessile, soft-bodied marine species such as macroalgae, sponges and soft corals (Fusetani, 2011; LimnaMol et al., 2011; Patro et al., 2012; Qian et al., 2015). Despite being exposed to surface biofouling in the marine environment, some of these species are able to remain free of fouling. As they lack the necessary behavioral or physical defenses, it is believed that they resist epibiosis via the production of antifouling secondary metabolites (Schmitt et al., 1998; Dobretsov et al., 2004, 2015). Numerous NPAs have been isolated from sessile, soft-bodied marine organisms, suggesting that they are promising sources of NPAs.

Soft corals are one of the most prolific sources of bioactive marine natural products. A variety of secondary metabolites have been extracted from soft corals, indicating various cytotoxic, antibacterial, anti-inflammatory, antiviral and antifouling bioactivities (Wen et al., 2008; Lai et al., 2013;Cheng et al., 2015; Gomaa et al., 2015; Taira et al., 2015). Soft corals of the genus *Sinularia* are particularly well-known for containing bioactive substances with interesting chemical structures, including diterpenes, sesquiterpenes, polyhydroxylated steroids and polyamine compounds (Kamel and Slattery, 2005; Chen et al., 2012).

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Table 1
Compounds **1**–**7** isolated from the soft coral *Sinularia flexibilis*.

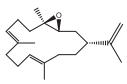
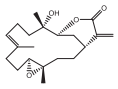
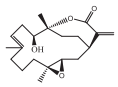
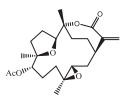
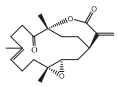
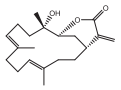
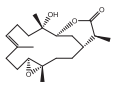
Compound	Name	Structure	Molecular weight	Property	Reference
1	Epoxycembrane A		288	Colorless oil	Bowden, (1981)
2	Sinularin		334	Colorless oil	Rymantas et al. (1978)
3	Sinulariolide		334	Colorless oil	Alfred and Weinheimer (1977)
4	(1*R*,13*S*,12*S*,9*S*,8*R*,5*S*,4*R*)-9-Acetoxy-5,8:12,13-diepoxycembr-15 (17)-en-16,4-olide		334	Colorless oil	Su et al. (2000)
5	11-Dehydrosinulariolide		332	Clear crystal	Liu et al. (2011)
6	(−)14-Deoxycrassin		318	Colorless oil	Wen et al. (2008)
7	Dihydrosinularin		336	Colorless oil	Alfred and Weinheimer (1977)

Table 2
^{1}H NMR and ^{13}C NMR Data for Cembrane diterpenes **1**–**7** (δ in ppm, *J* in Hz).

Position	1		2		3		4		5		6		7	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		40.4		33.7		35.1	3.21 m	35.7		34.9		33.4		36.9
2		33.6		22.6		31.4		33.2		32.6	1.42 m	26.3		27.7
3	2.83 dd (2.0,10.0)	63.2	3.97 d (11.0)	82.7		33.5		29.8		33.3	4.04 brd (11.0)	83.2	4.01 brd (8.0)	83.6
4		60.7		74.1		87		88.2		90.2		74.2		74.5
5		23.7		38.6	4.10 d (10.0)	68.7	4.38 dd (4.0,10.0)	83.4		209.3		39.5		38.1
6		38.3		27.8		35.8		26.7		30.1		22.7		23
7		123.9	5.23 t (11.0)	125.6		27		34.9		29.7	5.06 m	126.1	5.23 t (7.0)	125.9
8		135.1		134.5		134.8		85.4		135.2		134.6		133.8
9	5.10 t (6.4)	39.6		35.9	5.15 brd (9.0)	126.7	5.17 d (9.2)	77.9	5.09 t (6.9)	122.8		40.1		35.7
10		24.4		25.3		35.8		27.3		33.7		24.8		25.4
11	5.10 t (6.4)	124.3	2.79 brd (6.5)	62.9		38.1		34.3		37.6	5.07 m	126.6	2.87 t (6.0)	62.9
12		133.3		59		60.4		60		60.7		132.1		58.9
13		34.7		34.7	2.93 brd (10.7)	63.9	3.41 d (3.8,10.7)	60.8	3.18 dd (10.76,11.0)	62.3		36.1		34.8
14		29.8		32.9		31.7		33.5		31		31.8		31.2
15		148.6		140.1		144.4		145.4		143.7		140.2		42
16	1.63 s	110.7		167.2		169		169.5		167.7		167.3		174.7
17	4.68 brs 4.63 brs	18.5	6.47 brs 5.69 brs	127.9	6.29 brs 5.42 brs	124.1	6.27 brs 5.43 brs	123.6	6.33 brs 5.50 brs	125.8	6.47 d (2.4) 5.67 d (2.4)	126.4	1.37 d (7.0)	16.4
18	1.24 s	17	1.44 s	24.7	1.34 s	16	1.25 s	29.3	1.44 s	16.2	1.41 s	24.9	1.44 s	25.1
19	1.62 s	15.8	1.66 s	15.2	1.61 s	22.8	1.16 s	17.1	1.66 s	24.5	1.58 s	14.7	1.61 s	17.4
20	1.59 s	17.2	1.31 s	15.4	1.20 s	15.8	1.24 s	16.4	1.31 s	17.3	1.64 s	13.9	1.23 s	15.4
Acetoxyl							2.06 s	171.1 21.1						

Table 3
The known bioactivity of cembrane diterpenes from *Sinularia flexibilis*.

Activity	Compounds	References
Cytotoxic	Sinulaflexiolides D and E, flexilarin D, flexibilisolide C, 11-dehydrosinulariolide, sinularin,11-epi-sinulariolide acetate, sinulariolide, 11-acetylsinuflexolide, sinuflexolide, dihydrosinuflexolide, sinuflexibilin, 14-deoxycrassin, dehydrosinulariolide, dihydrosinularin, 3,4:8,11-bisepoxy-7-acetoxycembra-15 (17)-en-1,12-olide	Duh et al. (1998); Hsieh et al. (2003); Wen et al. (2008); Lin et al. (2009); Su et al. (2009); Shih et al. (2012); Su et al. (2013)
Anti-inflammatory	Flexibilisolides C and D, 11-dehydrosinulariolide, flexilarin D, (−)-sandensolide, 11-epi-sinulariolide acetate, isosinulaflexiolide K, sinulariolide, 11-epi-sinulariolide, sinulaflexiolide K, flexibilisquinone, 3,4:8,11-bisepoxy-7-acetoxycembra-15 (17)-en-1,12-olide	Shih et al. (2012); Y.F. Lin et al. (2013); Tsai et al. (2015)
Antimicrobial	Sinulariolide, flexibilide	Acerte et al. (1998)
Algacidal	Sinularin, 11-epi-sinulariolide	Michalek and Bowden (1997)

The soft coral *Sinularia flexibilis* (Alcyoniidae) keeps its body surface clean of macroepibionts. In this study we investigated the hypothesis that the seven metabolites we isolated from *S. flexibilis* are involved in its chemical defense against fouling. The antifouling effects of these compounds were tested against the larval settlement of two fouling organisms, the bryozoan *Bugula neritina* (Bugulidae) and the barnacle *Balanus albicostatus* (Balanidae). The former is a troublesome soft fouler with a cosmopolitan distribution, while the latter is a notorious hard fouler occurring in East Asian marine environments.

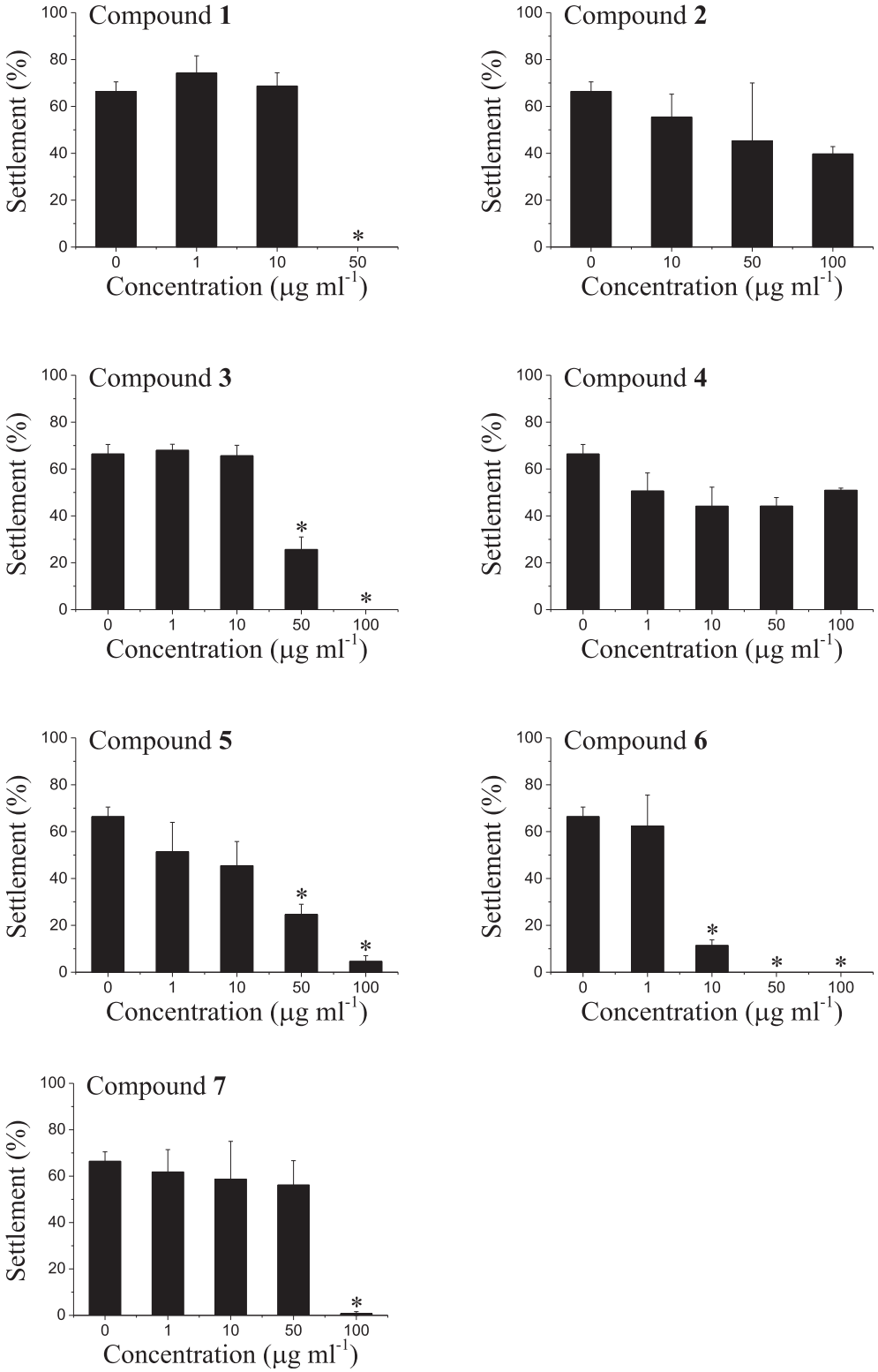


Fig. 1. Effects of compounds **1**–**7** on settlement of *Bugula neritina* larvae. Data shown are the means + SE of three replicates. Asterisks above the bars indicate data that are significantly different from the control (one-way ANOVA, Dunnett's test, $P < 0.05$).

2. Materials and methods

2.1. Collection, extraction and isolation

Specimens of *S. flexibilis* were collected by diving at a depth of 5–10 m in Sanya Bay (109°64′ E, 18°21′ N), Hainan Island, China in September 2011. The samples were extracted with methanol (MeOH) three times and the combined extracts were fractionated between dichloromethane (CH_2Cl_2) and water. The CH_2Cl_2 layer was subsequently subjected to elution by column chromatography (Toyopearl HW-40) using a CH_2Cl_2:MeOH (v/v, 2:1) mixed solvent system to obtain four fractions (fractions 1–4). Fraction 1 (68.4 mg) was chromatographed by High Performance Liquid Chromatography (HPLC; octadecylsilyl silica gel, MeOH:H_2O, v/v, 70:30, 1.5 ml/min, 254 nm, rt 99.7 min, 113.0 min) to yield compound **1** and compound **6**. Fraction 3 was separated by HPLC (silica gel, petroleum ether (PE):ethyl acetate (EtOAc), v/v, 7:3, 1.5 ml/min, 254 nm, rt 38.6 min, 43.0 min and 87.8 min) to produce compound **2**, compound **3** and compound **7**. Fraction 4 was chromatographed by HPLC (Octadecylsilyl silica gel, MeOH:H_2O, v/v, 95:5, 1.5 ml/min, 254 nm, rt 98.0 min, 120.1 min) to yield compound **4** and compound **5**. The structures of the compounds were elucidated based on NMR and MS spectral data. The NMR spectra were measured in deuterochloroform ($CDCl_3$) on a Bruker AVANCE 400 instrument (^{1}H-NMR, 400 MHz; ^{13}C-NMR, 100 MHz) using tetramethylsilane as an internal standard in both instances. The MS data were obtained using a Varian 7.0 T electrospray ionization mass spectrometer (ESI-MS; Varian, Inc. Palo Alto, CA, USA).

2.2. Assay for antifouling activity against the larval settlement of the bryozoan B. neritina

Adult *B. neritina* samples were collected from a fish farm located in Zhangzhou, Fujian Province, China. The larvae were obtained from the adults as described by Feng et al. (2013). Settlement assays using the larvae were conducted according to the method described by Qi et al. (2008), with some modifications. The purified compounds from the soft coral *S. flexibilis* were dissolved in dimethylsulfoxide (DMSO). A total of 20 μl of each compound solution and 1.98 ml sterile filtered seawater (0.22 μm, FSW) were added into each well of a 24-well plate, after which approximately 30 *B. neritina* larvae were added to each well. A 0.1% (v/v) solution of DMSO in FSW was used as a control. Three replicates were performed for each treatment and the control. The plates were incubated at room temperature in darkness for 24 h, after which the number of settled larvae were counted using a stereomicroscope and expressed as a percentage of the total number of larvae in each well.

2.3. Assay for antifouling activity against the larval settlement of the barnacle B. albicostatus

Adult samples of *B. albicostatus* were collected from the intertidal zone in Xiamen, Fujian Province, China. In the laboratory, the naupliar larvae were collected following their release from the adults upon submersion in seawater. The nauplii were then reared to cyprids according to the method described by Feng et al. (2009) and used in the settlement assays. The procedure for the settlement assays with *B. albicostatus* was similar to that used with *B. neritina*, as described above. Briefly, the compounds of interest were dissolved in DMSO. Next, approximately 30 cyprids, 20 μl of test solution and 1.98 ml FSW were added to each well of a 24 well plate. There were three replicates for each treatment and the control (0.1% DMSO in FSW, v/v). Following 48 h incubation in darkness at room temperature, the numbers of larvae that settled or died were enumerated using a stereomicroscope.

2.4. Statistical analysis

Differences in larval settlement or mortality between treatments and the control were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Significance was evaluated at the 5% level ($p < 0.05$). The concentration of the compounds that inhibited settlement by 50% relative to the control (EC_{50}), and the concentration that resulted in 50% mortality (LC_{50}), were calculated using the Spearman-Karber method (Hamilton et al., 1977, 1978; Reichelt-Brushett and Michalek-Wagner, 2005).

3. Results and discussion

In this study, seven known compounds were isolated from the soft coral *S. flexibilis* (Table 1). Their ^{1}H NMR and ^{13}C NMR data are listed in Table 2. Their chemical structures were identified based on their spectral data and comparison with those in the literature (Table 1). These compounds are all cembrane diterpenes containing a characteristic 14-membered carbocyclic skeleton. The genera *Lobophytum*, *Sarcophyton* and *Sinularia* have been reported to contain a rich diversity of cembrane diterpenes (Kamel and Slattery, 2005; Lakshmi and Kumar, 2009; Blunt et al., 2013; Lai et al., 2013; Nguyen et al., 2014; Nermeen et al., 2014). The seven compounds isolated in this study have been found in the above genera of soft corals; however, this is the first report of the isolation of epoxycembrane A (compound **1**) from *S. flexibilis*.

The previously reported bioactivity of cembrane diterpenes from *S. flexibilis* was summarized in Table 3. Here we tested the antifouling activity of compounds **1**–**7** against the bryozoan *Bugula neritina* and the barnacle *Balanus albicostatus*. Compounds **2** and **4** displayed no significant effect on larval settlement in *B. neritina*, while the other five compounds showed significant inhibitory activity, of which compound **6** was the highest (EC_{50} 3.90 μg ml^{-1}) (Fig. 1 and Table 4). Of the seven cembrane diterpenes, only compounds **1**, **3**, **4** and **6** had EC_{50} values below 50 μg ml^{-1} against barnacle settlement (Fig. 2 and Table 4). In addition, the LC_{50} values of compounds **1** and **4** were near their EC_{50} values in assay with barnacle (Table 4), indicating they might inhibit barnacle settlement through toxic mechanisms (Rittschof, 2011). Interestingly, compound **4** showed no antifouling activity in the bioassay with *B. neritina*, while significant antifouling activity was observed with *B. albicostatus* (Table 4). Furthermore, the EC_{50} value of compound **5** against *B. neritina* settlement was 21.02 μg ml^{-1}, while that against barnacle settlement exceeded 100 μg ml^{-1} (Table 4), suggesting species-specific effects of compounds on marine fouling organisms. This emphasizes the need to use more than one fouler as a target when evaluating the antifouling activity of compounds.

It has been suggested that soft corals exert a chemical response

Table 4
Antifouling activity of compounds **1**–**7** against the bryozoan *Bugula neritina* and the barnacle *Balanus albicostatus*.

Compound	*Bugula neritina*	*Balanus albicostatus*	
	EC_{50} (μg ml^{-1})	EC_{50} (μg ml^{-1})	LC_{50} (μg ml^{-1})
1	21.37 (21.25–21.50)	30.60 (29.93–31.29)	36.66 (35.86–37.47)
2	>100	>100	>100
3	33.18 (32.77–33.61)	21.00 (20.82–21.19)	61.27 (59.58–63.02)
4	>100	20.34 (20.13–20.55)	33.23 (32.56–33.91)
5	21.02 (20.16–21.92)	>100	>100
6	3.90 (3.84–3.97)	21.26 (21.13–21.39)	>100
7	55.60 (54.56–56.65)	>50	>50

The data are expressed as EC_{50} (or LC_{50}) and 95% confidence limits.

in competition for space, as well as against predators and foulers (Khalesi et al., 2008). The aqueous extract of *S. flexibilis* was previously shown to have antifouling activity by Maida et al. (2006). Additionally, sinularin (compound **3**) and 11-epi-sinulariolide from *S. flexibilis* have been found to have algacidal properties (Michalek and Bowden, 1997). To the best of our knowledge, the present study provides the first laboratory evidence of the antifouling activity of compounds **1** and **4**–**7** (although the majority of them have

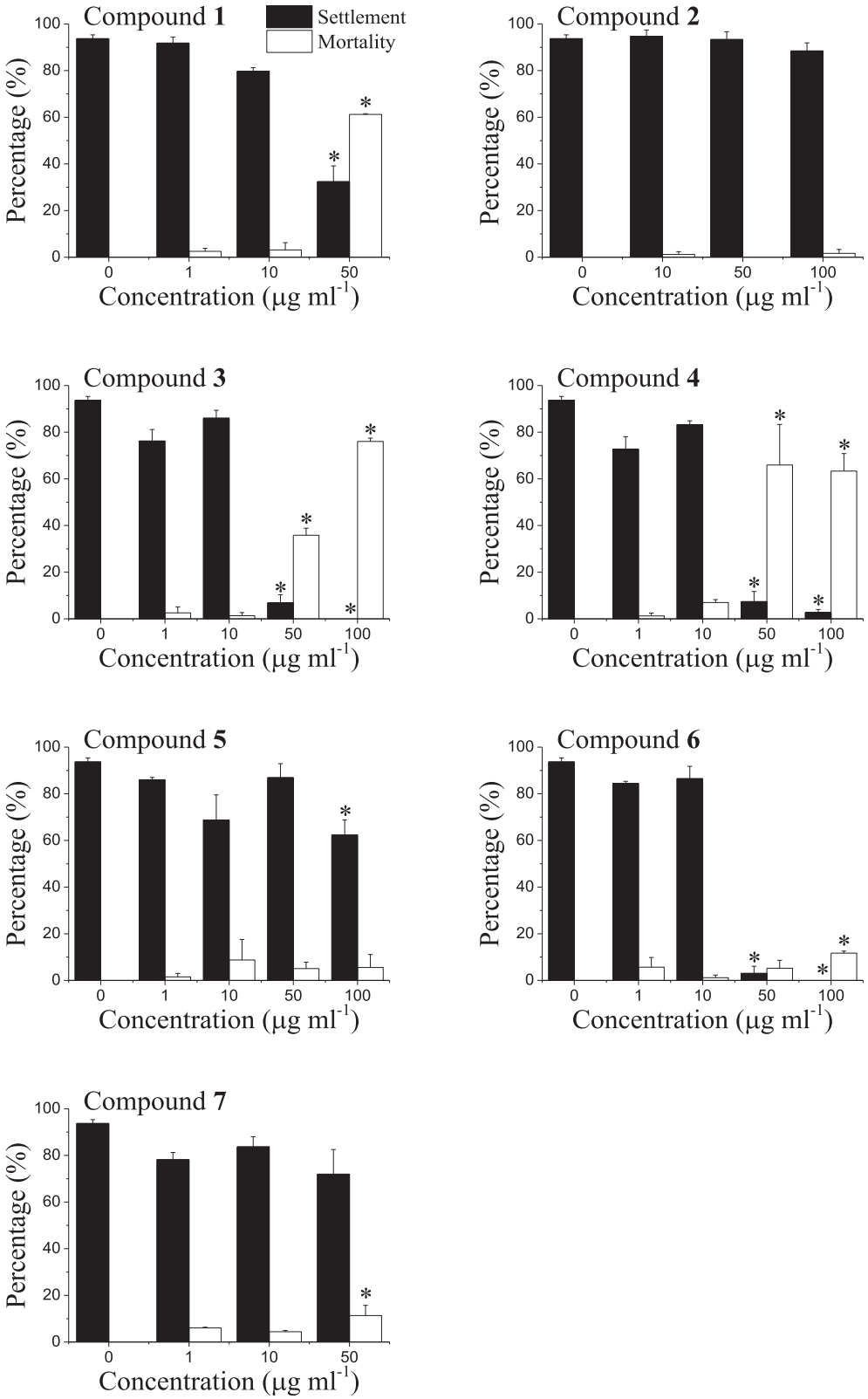


Fig. 2. Effects of compounds **1**–**7** on settlement (*black bar*) and mortality (*white bar*) of *Balanus albicostatus* larvae. Data shown are the means + SE of three replicates. Asterisks above the bars indicate data that are significantly different from the control (one-way ANOVA, Dunnett's test, $P < 0.05$).

previously been reported to have antitumor or anti-inflammatory properties by Martin and Uriz, 1993; Chen et al., 2012; Shi et al., 2012; Liu et al., 2011), suggesting their involvement in the chemical defense against fouling in *S. flexibilis* and possibly also other soft corals.

In this study, compound **6** was particularly significant as it exhibited remarkable antifouling activity against two fouling species, as well as low toxicity (LC_{50} > 100 μg ml^{-1}). This compound was previously found to possess anti-inflammatory activity by reducing the expression of two pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), in an assay using lipopolysaccharides (LPS)-induced RAW264.7 macrophage cells (C.Y. Lin et al., 2013). Furthermore, this compound was reported to have an immunomodulatory effect by decreasing the production of the immunomodulatory cytokine tumor necrosis factor (TNF)-α in an assay using LPS-stimulated marrow-derived dendritic cells (Su and Wen, 2011). The mechanism of antifouling activity of compound **6** requires further investigation. The present work confirms its potential use as an environmentally friendly antifoulant. The limited availability of natural antifouling compounds is a major obstacle hindering their development into commercial products. Hence, it is encouraging that *S. flexibilis* has been successfully cultured in small-scale trials (Khalesi et al., 2008, 2009). Furthermore, the chemical synthesis of cembrane diterpenes has been addressed by several studies (Lan et al., 2000; Rodney and Arun, 2011). In conjunction, the results of these studies may contribute towards alleviating the limited supply of (−) 14-deoxycrassin.

In conclusion, seven cembrane diterpenes were isolated from the soft coral *S. flexibilis* and six of them were found to be antifouling active. Compound **6**, (−)14-Deoxycrassin, with high antifouling activity and low toxicity, proved to be a candidate for a novel antifoulant.

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